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Purification and Nature of the Antibiotic Nisin

By N. J. BERRIDGE
National Institute for Research in Dairying, University of Reading

and G. G. F. NEWTON and E. P. ABRAHAM
Sir William Dunn School of Pathology, University of Oxford

(*Received* 12 *February* 1952)

The production of nisin by a strain of *Streptococcus lactis* (Lancefield group N) was described by Mattick & Hirsch (1944, 1947). Berridge (1949) obtained the antibiotic in purified form by fractional precipitation with salt and 'crystallization' from 80 % (v/v) ethanol. This product was found to yield alanine, valine, leucine and isoleucine, and possibly cystine and aspartic acid, on hydrolysis. A solubility curve, however, showed that it contained at least two substances, the less soluble of which amounted to about two-thirds of the whole.

Hirsch (1951) studied the relative antibacterial activity of three batches of nisin under various conditions and concluded that he was dealing with several antibiotics. Berridge (1952) investigated the behaviour of several preparations of nisin on counter-current distribution in twenty tubes. He obtained evidence that some of the preparations contained two active components, although the resolution was not sufficient to permit a confident analysis of the curve. Bavin, Beach, Falconer & Friedmann (1952) stated that a counter-current distribution curve of nisin showed two active peaks.

The aim of the work described in the present paper was to resolve a single batch of nisin into its components, and to isolate these components in quantities sufficient for some of their biological and chemical properties to be studied. The antibacterial properties of the components obtained are described by Gowans, Smith & Florey (1952).

RESOLUTION OF NISIN INTO SEVERAL SIMILAR POLYPEPTIDES

Methods

Solvent system. The resolution was carried out in a single experiment by counter-current distribution in a 102-tube all-glass apparatus (Craig, Hausmann, Ahrens & Harfenist, 1951). The starting material was a preparation of nisin purified by Bengers Ltd. (batch 706 P). Preliminary experiments showed that nisin had an overall partition coefficient close to unity in a solvent system consisting of methanol, *n*-butanol and acetate buffer at pH 3·0. Although nisin was not very soluble in it, this system was used for two reasons. First, because the starting material distributed between the two phases in approximately equal concentrations: other things being equal the maximum resolving power is obtained from a limited number of transfers when the geometric mean of the partition coefficients of the components of a mixture approaches unity (Craig & Craig, 1950). Secondly, because the pH of the system was one at which some groups in the material appeared to be partly ionized: differences in solubility due to differences in the dissociation constants of ionizable groups may occur under such conditions. A system in which nisin was more soluble could readily have been obtained by lowering the pH, but it would not have had these two advantages. Moreover, nisin was particularly stable in the solvent used. A number of solutions which were allowed to stand at room temperature for a month showed no loss in antibacterial activity.

Estimation of material in different fractions. The low solubility of nisin in the solvent system made it impracticable to determine the amount of material in the various fractions by weighing. The amount was determined in two ways: (1) by measuring total N by the micro-Kjeldahl procedure, and (2) by measuring the colour obtained when the mixture of amino-acids liberated on hydrolysis was treated with ninydrin in the manner described by Moore & Stein (1948). Reasonable agreement was found between the two sets of determinations.

Activities were measured by the turbidimetric method of Berridge & Barrett (1951).

Procedure. The 102 tubes of the machine were arranged in two rows of 51, one above the other, and a recycling device was fitted between tube 101 and tube 0 to enable the solutes to make a second run through the apparatus (Craig *et al.* 1951). The starting material was loaded equally into the first five tubes of the apparatus, and the fundamental procedure used until the leading upper layer was adjacent to tube 0. At this stage a single withdrawal of lower layers from the trailing edge was begun. Fresh lower layers were placed in the tubes thus left empty and the leading upper layers were recycled over them. By this procedure the nisin was subjected to 150 transfers.

Analysis of distribution curves. Values for the partition coefficients of the main components were obtained by examining successive points along the distribution curve.

Williamson & Craig (1947) showed that after n transfers have been made with a single substance placed initially in tube 0 the relationship between the fraction T_r of the substance in tube r, the fraction T_{r+1} in tube $r+1$, and the partition coefficient K, is given by

$$\frac{T_r}{T_{r+1}} = F'\left[\frac{1}{K}\right], \quad \text{where} \quad F' = \frac{(r+1)}{(n-r)}. \qquad (1)$$

When more than one tube is loaded at the beginning of the distribution this expression needs modification. Suppose that the number of tubes loaded is x. The final distribution

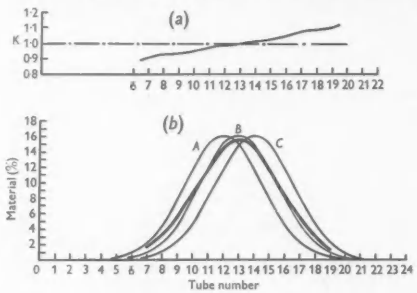

Fig. 1. (*a*) Values of K found from equation 2 (see text). (*b*) Effect of loading a substance with $K=1$ into the first three tubes before a 24-transfer distribution. Thin-lined curves A, B and C: distribution of the material initially in tubes 0, 1 and 2, respectively. Thick-lined curve: distribution of the total material which would be found experimentally.

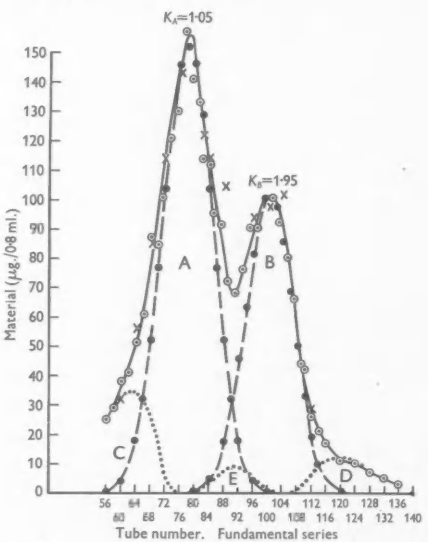

Fig. 2. 150-transfer distribution of nisin (preparation 706 P) in a solvent system consisting of acetic acid, sodium acetate, pH 3·0, methanol, and *n*-butanol. ⊙—⊙, μg. material/0·8 ml. of mixed layers estimated colorimetrically; ×—×, μg. material/0·8 ml. of mixed layers, estimated by the micro-Kjeldahl method; ●--●, calculated curves; ....., approximate interpretation for remaining material.

curve can then be regarded as the sum of a series of x separate curves, all having the same shape but each advanced one tube in front of its successor. If the separate curves are nearly symmetrical their summation will yield a curve whose maximum is midway between the separate maxima at each end of the series (Fig. 1). This curve cannot be described exactly as a simple function of n, T_r, T_{r+1}, and K, but its maximum will be in the same position as that of the curve derived solely from material loaded into tube $\frac{1}{2}(x-1)$, and if x is small and n large it will fit the latter rather closely.

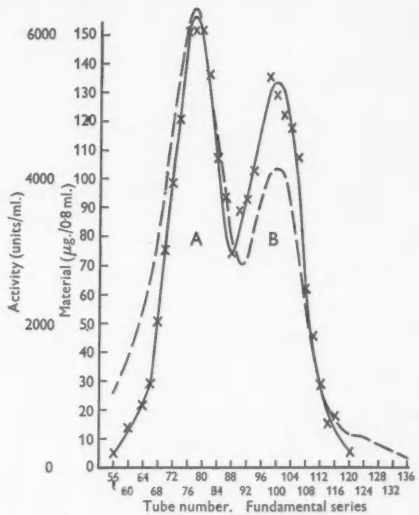

Fig. 3. 150-transfer distribution of nisin (706 P) in a solvent system consisting of acetic acid-sodium acetate, pH 3·0, methanol, and *n*-butanol. ×—×, antibacterial activity/ml. determined turbidimetrically; — — —, curve showing total material from Fig. 2.

The curve derived from material loaded into tube $\frac{1}{2}(x-1)$ is described by the equation

$$\frac{T_r}{T_{r+1}} = F'\left[\frac{1}{K}\right], \quad \text{where} \quad F' = \frac{[r+1-\frac{1}{2}(x-1)]}{[n-r+\frac{1}{2}(x-1)]}. \qquad (2)$$

Equation 2 can therefore be used for finding K when material is loaded into x tubes. If the material is homogeneous, and x is small and n large, the values obtained from different parts of the curve will show only minor variation; but they will be slightly larger on the leading side of the curve than on the trailing side, and will coincide with the true value at the maximum (Fig. 1).

Values of K were determined in this way over what appeared to be homogeneous portions of the curve, and these values were used for constructing sets of five separate theoretical curves each advanced one tube in front of the other. The curve obtained by summation of the separate theoretical curves could be fitted closely to the curve determined by experiment.

RESULTS

Counter-current distribution. The distribution curve shown in Fig. 2 indicates that the preparation of nisin was a complex mixture containing 49 % A,

32 % B, 12 % C, 5 % D and possibly 3 % of another substance E. Fig. 3 shows the distribution of activity.

Table 1, which should be compared with Fig. 2, shows the fractions that were selected for pooling and reconcentration. The specific activity of A against the standard test organism was similar to that of B and about five times that of C and D. The antibacterial properties of the concentrates were studied further by Gowans *et al.* (1952).

AMINO-ACID COMPOSITION OF THE NISIN POLYPEPTIDES

Nisins A, B, C and D

Fig. 4 shows two-dimensional paper chromatograms obtained from acid hydrolysates of nisins A, B, C and D. The chromatograms were developed first with butanol-acetic acid (Woiwod, 1949) and subsequently with phenol in the presence of the vapour of acetic acid. The chromatograms of A, B and C are strikingly similar, and only slight differences are apparent in that of D. An examination of the results has shown that leucine and/or isoleucine, alanine, glycine, proline, aspartic acid, histidine and lysine are present in all four compounds. Valine and methionine are present in A, B and C but are probably absent from D. The presence of histidine was confirmed by the Pauly reaction, and that of methionine by oxidation with hydrogen peroxide to the sulphone. Tryptophan was shown to be absent by the low absorption of nisin at 275 mμ. and by the

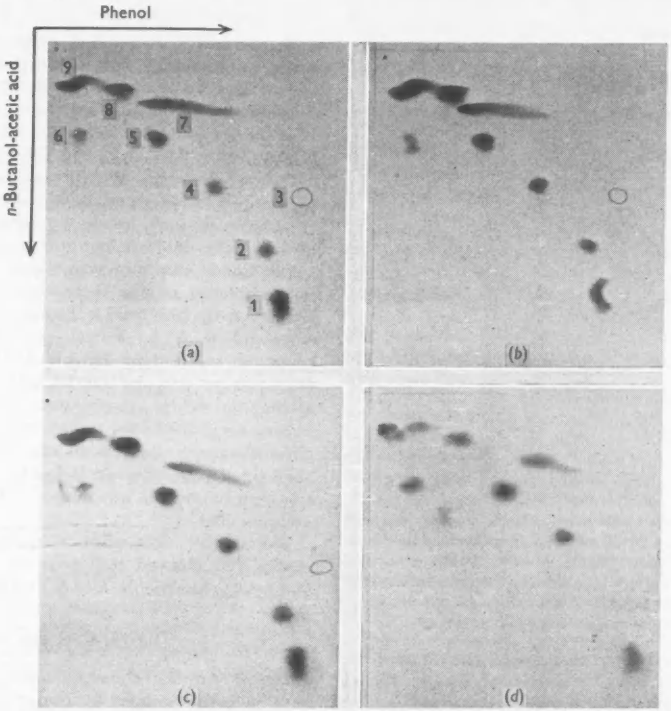

Fig. 4. Two-dimensional paper chromatograms of nisins A, B, C and D. (*a*), A; (*b*), B; (*c*), C; (*d*), D. Spots developed with ninhdyrin: (1) leucine and/or isoleucine; (2) valine and methionine; (3) proline; (4) alanine; (5) glycine; (6) aspartic acid; (7) lysine and histidine; (8) cystathionine; (9) lanthionine.

Table 1. *Antibacterial activities of the concentrates prepared from the pooled fractions obtained after counter-current distribution*

	Tube numbers (inclusive)		Units* of antibacterial activity/mg. against *Strep. agalactiae*
	Fundamental series	Withdrawn series	
A	76–81	—	32 000
B	102–108	—	41 500
C	56–62	54–40	10–5 000
D	116–140	—	10–5 000
E	90–94	—	Not known

* The unit is that defined by Berridge (1949).

fact that it showed no colour when treated with Ehrlich's reagent.

The four nisins also contain two substances which react with ninhydrin but which are not normally found in protein hydrolysates. These are shown as spots 8 and 9 in Fig. 4. After treatment of the hydrolysate with hydrogen peroxide they appeared as a double spot close to the origin. Further information about the nature of these substances was obtained by hydrogenolysis. A mixture of the amino-acids in spots 8 and 9, which

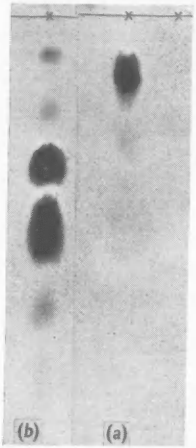

Fig. 5. One-dimensional paper chromatogram run with butanol-acetic acid of material from spots 8 and 9 together (see Fig. 4). Spots developed with ninhydrin. (*a*) Untreated material; (*b*) the same material after treatment with Raney nickel. The major spots in (*b*) are in the positions of alanine and α-amino-*n*-butyric acid.

had been eluted from a series of one-dimensional chromatograms, was treated with Raney nickel. The products of the hydrogenolysis were found by paper chromatography to be alanine and α-amino-*n*-butyric acid in about equal amounts (Fig. 5). Hydrogenolysis of 1 part of lanthionine and 2 parts of cystathionine yielded a mixture which showed the same amino-acid pattern, although it contained a smaller proportion of α-amino-*n*-butyric acid. These results indicated that lanthionine and cystathionine or *allo*cystathionine might be present in the mixture.

To simulate a nisin hydrolysate, a mixture was made of the following amino-acids in simple proportions by weight: leucine (4), valine (2), alanine (2), glycine (2), proline (2), serine (1), lysine (2), histidine (2), aspartic acid (1), methionine (2), lanthionine (1), and cystathionine (4).

The pattern formed by this mixture in two-dimensional paper chromatograms did in fact reproduce very closely that formed by a nisin hydrolysate, both before and after treatment with hydrogen peroxide. Moreover, no new spots were seen after chromatographing a nisin hydrolysate to which lanthionine and cystathionine had been added.

Nisin D would appear to contain some of the sulphur-containing amino-acids in the oxidized form (Fig. 4).

Amino-acid composition of different batches of nisin

Hydrolysates of various preparations of nisin, made commercially during the period of 1948–51, were compared on one-dimensional chromatograms developed with butanol-acetic acid. Eight batches of nisin prepared by Bengers Ltd. during 1948–9 (652A, 660P, 33Y, XP, 696, 754, 706P, EPS 756) contained the amino-acids present in nisins A, B and C. Batch 696 contained, in addition, glutamic acid. A sample of nisin made by Genatosan in 1949 (batch 263) did not contain glutamic acid but significant amounts of serine were present. Batch 263 was used in the therapeutic experiments of Gowans *et al.* (1952) and is the material referred to as 'preparation A' by Hirsch (1951). Of the other preparations used by Hirsch (1951) and Berridge (1952) only 'preparation L' and batch 138 were available. When these were hydrolysed and chromatographed they were both found to contain glutamic acid and serine in addition to the amino-acids present in nisins A, B and C. All the preparations contained the uncommon sulphur-containing amino-acids.

Elementary analysis of nisin hydrochloride (batch 263) showed the presence of 5·6 % sulphur and 5·5 % chlorine (Weiler & Strauss).

DISCUSSION

It is now clear that the name nisin must be regarded as a collective one for a number of closely related polypeptides. In this respect nisin resembles several other antibiotics obtained from bacteria. For example the gramicidins, tyrocidines (Craig, Gregory & Barry, 1949), licheniformins (Callow & Work, 1952) and bacitracins (Newton & Abraham, 1950, 1952) occur as families of similar peptides.

The main active polypeptides in the single preparation of nisin that was resolved by counter-current distribution resembled each other very closely in amino-acid composition. Other batches of nisin that had not been purified by counter-current distribution also contained a similar series of amino-acids, although glutamic acid and serine were present in some preparations but not in others.

The nisin polypeptides are remarkable for their high content of sulphur. In addition to methionine, which is commonly found in proteins, they appear to

contain lanthionine and a considerable amount of cystathionine.

Lanthionine has been isolated from acid hydrolysates of wool that has been treated with alkali (Horn, Jones & Ringel, 1941). It is assumed to be formed, on alkali treatment, from cystine residues in the protein, although it has been detected in small amounts in an acid hydrolysate of the tips of virgin wool (Consden, Gordon & Martin, 1946).

Cystathionine has been isolated from the mycelium of a certain strain of *Neurospora* and has been shown to be an intermediate in the biosynthesis of methionine (Horowitz, 1947). It appears to have also been obtained from wool treated with sodium sulphide (Küster & Irion, 1929), but there have been no reports of its occurrence in a native protein.

Although lanthionine occurs rarely, if at all, in proteins, it is present in the antibiotic subtilin, a polypeptide produced by a strain of *Bacillus subtilis* (Dimick, Alderton, Lewis, Lightbody & Ferrold, 1947). Lewis & Alderton (1948) found that subtilin contained 4·8% sulphur, and that on acid hydrolysis it yielded lanthionine and an amino-acid with the empirical formula $C_7H_{14}O_4N_2S$. It seems not unlikely that the latter was a cystathionine or *allo*-cystathionine.

Nisin and subtilin show some general similarities in their amino-acid composition and also in their physical and antibacterial properties. However, the two substances are clearly distinct. Nisin contains no tryptophan and subtilin no methionine.

EXPERIMENTAL

Solvents

'Analar' glacial acetic acid, *n*-butanol of laboratory reagent grade and commercial refined methanol (B.S.S. no. 506 (1950), Imperial Chemical Industries Ltd.) were used without further purification.

Estimation of material

(*a*) *Micro-Kjeldahl.* The procedure of Tompkins & Kirk (1942) was used, with the digestion mixture of Campbell & Hanna (1937) and a digestion period of 40–50 min.

(*b*) *Photometric ninhydrin.* The method described by Newton & Abraham (1950) was used. The colour obtained from nisin after hydrolysis was very close to that obtained from an equal weight of leucine. Samples of 2 ml. volumes were taken from each layer of the contents of the even-numbered tubes of the fundamental series, and 3 ml. samples from the single layer of the withdrawn series. Estimations on the odd-numbered tubes 71–109 inclusive were also made in an independent experiment.

Measurement of antibacterial activity

The turbidimetric method of Berridge & Barrett (1951) was used. By using a syringe for making serial dilutions, an automatic pipette for measuring volumes, and a culture of the test organism in its logarithmic phase of growth, this method enabled a large number of assays to be completed in replicate, simultaneously, and in a few hours. Some causes of variation were thereby eliminated and an improvement in accuracy was obtained.

Counter-current distribution

Solvent system. The mixture was made by adjusting a solution of 400 ml. of glacial acetic acid in 3200 ml. of water to pH 3·0 with concentrated NaOH, and adding 400 ml. of methanol and 2000 ml. of *n*-butanol. Such a mixture, after equilibration, gave a pH reading with the glass electrode of 3·56 for the lower phase and 3·52 for the upper phase. The quantity of NaOH needed was very low, pH 3·0 being near the beginning of the titration curve of acetic acid. The solvent was kept no longer than overnight to reach equilibrium before use.

Distribution procedure. The fixed volume of the stationary lower layer was 20 ml. The machine was filled with solvent, as described by Craig *et al.* (1951). The contents of tubes 0–4 inclusive were then removed and to each of these tubes were added 60 mg. of purified nisin dissolved in a mixture of 20 ml. top phase and 20 ml. bottom phase. Altogether, 300 mg. of nisin were therefore used in the distribution.

The fundamental procedure for operating the machine (Craig & Post, 1949) was then carried out, using 20 ml. quantities of upper layer, until the upper layer which was originally in tube 4 had reached tube 99. At this stage a fresh upper layer was not put into tube 0. A further transfer was then made and the contents of tube 0 (a lower layer only) were removed as the first member of the withdrawn series. Tube 0 was left empty. The cycle of operations was repeated and the contents of tube 1 were withdrawn. The 'front' of the mobile phase was now in tube 101, the last tube of the bottom row of the machine. Therefore an excess of lower layer (about 40 ml.) was put in tube 0 so as to provide a fresh lower layer for the first step of the recycle. After the next transfer and withdrawal an increment of fresh lower layer was added to tube 1. This cycle of operations was continued until the 'front' of the upper layers had reached tube 52 for the second time. All the material which was originally put into the machine had now completed 150 transfers.

Concentration of pooled samples. In each case HCl was added to the mixture until the pH reading fell to $1·9 \pm 0·1$, and the solution was then concentrated *in vacuo* with a bath temperature of 50°. The apparent pH was maintained between 1·8 and 2·0 by periodic adjustments, from four to six interruptions being necessary during the concentration of each batch.

In the absence of butanol frothing was very troublesome, so that on occasions when all the butanol had evaporated before the end of the concentrating, extra butanol was added when the pH was adjusted. In this way 10- to 20-fold concentration was readily achieved.

To each concentrate were added 40 vol. of acetone (the amount usually used in preparation work is 10 vol.). The resulting precipitate of nisin and NaCl was collected, by decantation and centrifuging, into a small tube and extracted four times with 1 ml. of 0·05 N-HCl. The NaCl dissolved first and, as nisin is of low solubility in saturated salt solution, the first extracts were of low activity. The four solutions were therefore bulked according to their assay values. The preparations were left as concentrated solutions in acid, since nisin is quite stable under these conditions,

and it is difficult to prepare dry powders without the risk of loss, particularly on such a small scale.

Minor variations of the above procedure were tried with less success.

Hydrolysis of nisin

The sample of nisin was sealed in a hard-glass tube with 6N-HCl (1 ml. of acid was used for a 10 mg. sample) and heated at 110° for 24 hr. The hydrolysate was freed from HCl by evaporation in an air stream at 100°.

Preparation of paper chromatograms

Whatman no. 1 paper was used, and 400 μg. of material were applied to the paper for each two-dimensional chromatogram. The chromatograms were developed at 21° with a butanol-acetic acid-water mixture (Woiwod, 1949). They were then dried in a current of air at 50–70° until most of the acetic acid had been removed. Phenol-water (4:1), in presence of the vapour of 50% (w/v) acetic acid was used as the second solvent (Dent, 1948). After the phenol had run, the papers were dried in a stream of warm air at less than 40°. The positions of the amino-acids were revealed by treatment with ninhydrin (Woiwod, 1949).

To oxidize the sulphur-containing amino-acids 30% (w/v) H_2O_2 was mixed with the sample before applying it to the paper (Dent, 1948).

Hydrogenolysis with Raney nickel

Raney nickel was prepared from nickel-aluminium alloy by the method of Mozingo, Wolf, Harris & Folkers (1943).

Preparation of α-amino-n-butyric acid

Methionine (200 mg.) was dissolved in water (15 ml.) and one spatula-full of Raney nickel was added to the solution. The mixture was heated under reflux in an oil bath at 120° for 3 hr. The Raney nickel was removed by centrifuging and washed with a small amount of water. Nickel was then removed from the solution by shaking three times with an equal volume of 8-hydroxyquinoline in $CHCl_3$. After shaking three times with an equal volume of $CHCl_3$, to remove traces of 8-hydroxyquinoline, the aqueous solution was evaporated to dryness. The residue (170 mg.) was dissolved in a small amount of hot water, and ethanol added until the solution was just turbid. On cooling, α-amino-n-butyric acid separated in crystalline form. It was shown by paper chromatography to be homogeneous.

Hydrogenolysis of lanthionine and cystathionine

A mixture of lanthionine (1 mg.) and cystathionine (1·6 mg.) was dissolved in 5 ml. water and a small spatula-full of Raney nickel added to the solution. The mixture was heated under reflux for 3 hr., and the Raney nickel removed at the centrifuge. The resulting solution was shaken with 8-hydroxyquinoline in $CHCl_3$ and then with $CHCl_3$ alone. The aqueous solution was evaporated to dryness and the residue chromatographed on paper with butanol-acetic acid.

Hydrogenolysis of spots 9 and 8

Twenty spots of hydrolysed nisin 263 (500 μg. each) were chromatographed in one dimension on a single sheet of paper. The chromatogram was developed with butanol-acetic acid (Woiwod, 1949). The paper was freed from solvent and then heated at 115° for 20 min. The positions occupied by the amino-acids on the paper could then be clearly seen in ultraviolet light. The area occupied by spots 8 and 9 were cut out and the amino-acids eluted from the paper by the method of Sanger & Tuppy (1951). A small spatula-full of Raney nickel was then added to the eluate and the mixture heated under reflux in an oil bath at 120° for 3 hr. The Raney nickel was removed by centrifuging and washed with a small amount of water. The supernatant was shaken with a solution of 8-hydroxyquinoline in $CHCl_3$ and then three times with $CHCl_3$ alone. The resulting aqueous solution was evaporated to dryness and the residues examined on a one-dimensional paper chromatogram developed with butanol-acetic acid (Fig. 5).

SUMMARY

1. A single preparation of nisin has been resolved into at least four active polypeptides by counter-current distribution between solvents. These substances have been named nisin A, B, C and D.

2. Nisins A and B show similar activity against *Streptococcus agalactiae*. Nisins C and D are about one-fifth as active as A and B.

3. Nisins A, B and C appear to contain leucine and/or isoleucine, valine, alanine, glycine, proline, aspartic acid, histidine, lysine, methionine, lanthionine and a cystathionine or *allo*cystathionine. Cystathionine appears to be present in larger amount by weight than most of the other amino-acids. Nisin D contains glutamic acid but no valine or methionine.

4. Various commercial preparations of nisin have been found to be similar in amino-acid composition, but some batches contain glutamic acid and serine, which are absent from nisins A, B and C.

5. The nisin polypeptides show some resemblance in their properties to the antibiotic subtilin.

We wish to record our thanks to Mr J. Barrett for assistance in assaying samples of nisin and to Mr Omar Boys whose help has been invaluable in running the paper chromatograms. We are grateful to Bengers Ltd. for gifts of nisin and to Mr S. Wijesundera and Mr F. Gibson of the Department of Microbiology, Oxford, for a gift of cystathionine.

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Studies in Vitamin A

19. PREPARATION AND PROPERTIES OF RETINENE$_2$

By H. R. CAMA, P. D. DALVI, R. A. MORTON, M. K. SALAH, G. R. STEINBERG and A. L. STUBBS

Department of Biochemistry, University of Liverpool

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Retinene$_2$ was first recognized by Wald (1937) and described as a 'deep yellow carotenoid' obtained from the retinas of freshwater fishes; when dissolved in chloroform it showed a wide absorption band with $\lambda_{max.}$ at 405 mμ., and in the presence of excess of the antimony trichloride reagent it gave a greenish blue colour with $\lambda_{max.}$ at 702–706 mμ.

The retinas of freshwater fishes are violet in colour (Kühne & Sewall, 1880), whereas those of most animals and sea fishes are pink. The spectral shift (Köttgen & Abelsdorff, 1896) is now ascribed (Wald, 1937, 1938–9*a*, *b*) to the partial or complete replacement of visual purple (rhodopsin) by porphyropsin with an absorption peak near 522 mμ. instead of 500 mμ.

Exposure to light liberates retinene$_1$ from rhodopsin and retinene$_2$ from porphyropsin, but the amounts obtainable from retinas are of course too small for other than spectroscopic identification. Retinene$_1$ has been shown to be vitamin A$_1$ aldehyde and can now be obtained in quantity by oxidation of vitamin A$_1$ alcohol (Ball, Goodwin & Morton, 1948). Similarly, retinene$_2$ is the aldehyde of vitamin A$_2$ (Morton, Salah & Stubbs, 1947), but its preparation in a state of purity has been difficult.

The term vitamin A$_2$ was first used by Edisbury, Morton & Simpkins (1937) on the strength of the very close analogy between the distribution of vitamin A$_1$ in the organs of marine fishes and the distribution of a '693 mμ. chromogen' in freshwater fishes. The antimony trichloride colour test applied to vitamin A$_1$ gives a blue solution ($\lambda_{max.}$ 620 mμ.), whereas when applied to vitamin A$_2$ it gives a greenish blue solution ($\lambda_{max.}$ 693 mμ.). The ultraviolet absorption peaks are at 325–328 mμ. for vitamin A$_1$ and 350–352, 286 mμ. for vitamin A$_2$. The 693 mμ. chromogen had been described earlier (Heilbron, Gillam & Morton, 1931). Its importance was clearly recognized by Lederer & Rosanova (1937) and Wald (1938–9*a*).

The problems to be discussed in this and succeeding papers have a rather complicated background which needs explanation.

(1) Fish-liver oils with a high vitamin A$_2$ content are not easy to obtain; most commercial oils are of marine origin and vitamin A$_2$, although frequently present, rarely accounts for more than 5–10% of the vitamin A content. For the British freshwater fishes which have been studied, the absolute amount of vitamins A present in liver oils is quite low and the

proportion as vitamin A_2 is approximately one-third to one-quarter of the total.

(2) There is at present no satisfactory method of effecting a clean separation of vitamin A_2 from vitamin A_1 and the best method of preparing retinene$_2$ is first to obtain a sterol-free concentrate of mixed vitamins A_1 and A_2 and then to oxidize the alcohols to a mixture of retinene$_1$ and retinene$_2$. These can then, with difficulty, be separated by chromatography. When the separation is practically complete, further chromatography removes artifacts, and low-temperature crystallization can be used to complete the purification. The retinene$_2$ can then be reduced to vitamin A_2 by means of lithium aluminium hydride.

(3) Retinene$_2$ and vitamin A_2 are very labile substances, particularly unstable when nearly pure and not easy to analyse for carbon and hydrogen. This is a matter of some importance because, as will be shown, much depends on whether the formula of retinene$_2$ is $C_{20}H_{28}O$ or $C_{20}H_{26}O$. Throughout the 6 years over which the present studies have been spread there has been uncertainty about the structural formula of vitamin A_2. The evidence has been so conflicting (see p. 539) that an approach via the structure of retinene$_2$ might be decisive. The dehydrovitamin A_1 formula for vitamin A_2 favoured by Morton *et al.* (1947) has recently been proved by synthesis (Farrar, Hamlet, Henbest & Jones, 1951) and the specification for retinene$_2$ (communicated to them) confirmed, because the synthetic retinene$_2$ obtained by the manganese dioxide oxidation (Ball *et al.* 1948) of synthetic vitamin A_2 agreed with natural retinene$_2$ in all respects.

(4) There are many possibilities of isomerism in vitamins A_1 and A_2 and therefore in the retinenes. If a mixture of neovitamins A_1 and A_2 and *all-trans* vitamins A_1 and A_2 is oxidized there may be four or more retinenes. Each of these is subject to the risk of epoxidation and the formation of other artifacts. Attempts to dehydrogenate vitamin A_1 to form retinene$_1$ and retinene$_2$ by the Oppenauer reaction give rise to a variety of products depending on experimental conditions.

For these various reasons much of the work has been repeated many times. The main results had been obtained 2½ years ago, but much labour has since been devoted to obtaining larger amounts of purified materials for analysis and complete characterization.

EXPERIMENTAL

Materials and methods

Sources of vitamin A_2. (*a*) Pike-liver oil. The livers of pike (*Esox lucius*) obtained from Lake Windermere (by courtesy of the Director, Freshwater Biological Research Station) were extracted by standard methods. The resulting oil was low in vitamins A, but A_2 predominated over A_1 ($E^{1\%}_{1\,cm.}$ 693 mμ. 0·52; 620 mμ. 0·18). The pike is by far the most convenient British freshwater fish to use as a source of vitamin A_2 although the yield is poor. (*b*) Ling-cod liver oil. Of all the marine fish-liver oils so far examined for A_2 that of ling cod (*Ophiodon elongatus*) is much the best. The total vitamin A content is high (sometimes 15%), but there is 7 to 10 times as much A_1 as A_2. Supplies of oil were obtained from the U.S.A. through the good offices of the Ministry of Food and of Parke Davis and Co. (*c*) Lake Nyasa oil. This oil from the livers of large fishes (species uncertain) is the best source known to us of vitamin A_2 ($E^{1\%}_{1\,cm.}$ 693 mμ., 174; 620 mμ., 530; A_2/A_1 approx. 1/3). We are indebted to Marine Oil Refiners of South Africa for help in obtaining the oil.

Manganese dioxide. Two kinds were used: (*a*) a fine grade of commercial laboratory reagent and (*b*) material obtained by mixing equivalent amounts of $MnSO_4$ (cryst.) and $KMnO_4$ in aqueous solution, filtering and washing free from SO_4^{2-} and drying on a porous plate in a desiccator. (Details of an improved preparation are given by Attenburrow *et al.* 1952.)

Light petroleum. In the main the commercial product (b.p. 40–60°) was used, but for spectrophotometry a material purified by stirring with H_2SO_4 (conc.), washing and distilling (b.p. 60–80°) was preferred.

Diethyl ether. In order to eliminate peroxides, ether was redistilled over reduced Fe before use.

Alumina for adsorption. Grade O alumina (Peter Spence and Co.) was weakened by incorporating known amounts of water, 5–10%, which were stirred in slowly under light petroleum. (Percentages of water added are w/w.)

Absorption spectra. These were determined using photoelectric instruments, except that routine colour-test determinations were made on a Hilger-Nutting visual spectrophotometer.

Preparation of retinene$_2$ from ling-cod liver oil

Preliminary. The oil used contained 8 times as much A_1 as A_2. Chromatography on the oil gave a fraction in which the ratio A_2/A_1 was raised to 1/3 but it contained only 5% of the vitamin used. Chromatography on the unsaponifiable fraction gave a better yield of enriched product, but the vitamin A_2 proved unstable, presumably because of the removal of natural antioxidants.

The sterol-free unsaponifiable fraction from the original oil was therefore oxidized over MnO_2 according to Ball *et al.* (1948). A portion of the solution of mixed retinenes was chromatographed on full strength alumina, but gave rise to red artifacts in undesirable quantities. Mixtures of various retinene$_1$ and retinene$_2$ derivatives (obtained with aldehyde reagents) could not be separated easily. Chromatography of oxidized unsaponifiable material on bone meal, magnesia or lime was ineffective or detrimental, but 10% watered alumina permitted appreciable separation.

Preparation of vitamin A_1 and A_2 concentrate. Unsaponifiable fractions were obtained from 100 g. portions of ling-cod liver oil and dissolved in methanol. A first crop of sterol separated overnight at 0°. The filtrate was cooled to −70° and a further crop of crystals removed by low-temperature filtration through a cooled, jacketed, Büchner funnel. The filtrate was diluted with one-third its volume of water and extracted with light petroleum (3 × 50 ml.). The solvent was removed and the residue dried by blowing with N_2 after small additions of ethanol. (Yield 8·8 g., $E^{1\%}_{1\,cm.}$ 617 mμ., 3300; 693 mμ., 500; A_2/A_1 approx. 1/6·5.)

Initial isolation of a 740 mμ. chromogen. The concentrate was dissolved in light petroleum (100 ml./g.) and the solution left to stand over MnO_2 (4 g./100 ml.) in the dark for 4 days with occasional shaking. The solution was then filtered and reduced to small bulk. The oxidized material (4 g.) was chromatographed on a column (20 × 2·5 cm.) of 10% watered alumina. Continuous development with light petroleum gave three ill defined zones; the column was extruded, sectioned, and eluted with $CHCl_3$. The middle zone was the richest in a material giving $\lambda_{max.}$ 740 mμ. in the colour test, and the retinene$_1$ band at 664 mμ. was shown at about one-quarter the intensity found before chromatography. The $CHCl_3$ was removed completely and replaced by light petroleum. The solution was chromatographed as before and the middle zone retained. Two maxima were seen in the colour test, the 664 mμ. peak being twice as intense as that at 740 mμ.

After a third chromatographic separation the middle zone showed the 740 mμ. chromogen predominating over the 664 mμ. chromogen. After a fourth separation a middle fraction showed only the 740 mμ. band ($E^{1\%}_{1\,cm.}$ 3000). After standing, the 740 mμ. colour-test maximum disappeared and a band with $\lambda_{max.}$ 705 mμ. persisted. The fraction when examined in $CHCl_3$ showed $\lambda_{max.}$ 405 mμ. and in *cyclo*hexane 385 mμ. The yield of retinene$_2$ free from retinene$_1$ was only 6 mg., but much retinene$_1$ could be obtained free from retinene$_2$.

Second isolation of retinene$_2$ from ling-cod liver oil. The unsaponifiable matter from 500 g. oil was obtained, partially freed from sterols and oxidized over MnO_2 (100 g.) in light petroleum (1 l.). The filtrate was chromatographed on fifteen columns (25 × 2 cm.) in portions of about 65 ml., on 10% watered alumina. The chromatograms were developed (3 hr.) with light petroleum and the middle portions retained, eluted and combined (yield 7·9 g.; in colour test $E^{1\%}_{1\,cm.}$ 740 mμ. 420; 664 mμ. 2070). This material (approx. 60% retinene$_1$, 10% retinene$_2$) was rechromatographed in two portions. The most strongly adsorbed portion contained 2·5 times as much retinene$_2$ as retinene$_1$. After the next adsorption the most strongly held portion showed approximately equal amounts of the two retinenes and a single absorption peak at 377·5 mμ. A further adsorption gave a fraction showing retinene$_2$ only ($\lambda_{max.}$ 385 mμ. in *cyclo*hexane, 740 mμ. fading to 705 mμ. in the colour test). The best fraction (0·11 g. from half the preparation) showed $E^{1\%}_{1\,cm.}$ 385 mμ. 1300. It was an orange-coloured viscous oil.

Preparation of retinene$_2$ from pike-liver oil

Pike-liver oil (300 g.), accumulated from several batches of pike livers, gave a sterol-free unsaponifiable fraction estimated to contain 75 mg. vitamin A_2. Oxidation over MnO_2 (7 days in darkness) gave a mixture of retinenes which after two adsorptions gave a fraction free from retinene$_1$. A third adsorption gave an upper fraction (4·5 mg.) and a middle fraction (20 mg.) showing $\lambda_{max.}$ 385 mμ. in *cyclo*hexane and 740 mμ. (fading to 705 mμ.) in the colour test. In addition to the main band at 385 mμ. there was an inflexion at 310 mμ.

Preparation of crystalline retinene$_2$

(1) *From ling-cod liver oil.* The unsaponifiable fraction from 800 g. was partially freed from sterols and oxidized as before; after four chromatographic separations 0·36 g. of crude retinene$_2$ free from retinene$_1$ was obtained ($E^{1\%}_{1\,cm.}$ 385 mμ., 900 in *cyclo*hexane). Part (0·24 g.) of this was dissolved in light petroleum (2 ml.) and slowly cooled to −70°. After standing overnight, the supernatant liquor was removed and the sticky deposit (m.p. 57°, $E^{1\%}_{1\,cm.}$ 1320) recrystallized. Recrystallization raised the melting point to 59° and then to 61°; $E^{1\%}_{1\,cm.}$ 385 mμ. 1460; no further rise in melting point was obtained by further recrystallization.

(2) *From ling-cod liver oil.* Several batches of oil were used to prepare crude retinene$_2$ as before, and a fraction showing $E^{1\%}_{1\,cm.}$ 1200 was crystallized from light petroleum (2% w/v). Three recrystallizations gave 30 mg. of retinene$_2$, m.p. 77–78°; solubility about 0·2% in light petroleum. The mother liquors gave a further yield of material of m.p. 77–78°. The analytical results will be discussed later. A third preparation from a new batch of ling-cod liver oil ($A_2/A = 10/1$) was undertaken. After repeated recrystallizations the melting point of retinene$_2$ was unchanged at 77–78° (corr.).

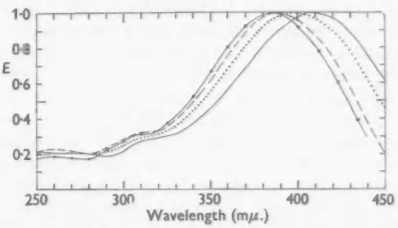

Fig. 1. Absorption spectrum of retinene$_2$ in light petroleum, ●—●; *cyclo*hexane, – – –; chloroform, ——; ethanol, ····· (shown as $E_{max.} = 1·0$ to illustrate solvent shifts; for absolute values see Table 1).

Table 1. *Spectroscopic properties of retinene$_2$ (m.p. 77–78°)*

Solvent	$\lambda_{max.}$ (mμ.)	$E^{1\%}_{1\,cm.}$	λ of inflexion (mμ.) (approx.)
Light petroleum (40–60°)	385	1490	305
*cyclo*Hexane	386	1440	310
Ethanol	397	1410	315
Chloroform	407	1360	320
*iso*Propanol	396	1440	315
$SbCl_3$ colour test	730–740 Fades to 705	4000*	—

* Initially higher, but after 15–20 sec. is steady between 3800 and 4000.

(3) *From Lake Nyasa oil.* This oil gave 35% of unsaponifiable material which after removal of sterols showed $E^{1\%}_{1\,cm.}$ 326 mμ. ~ 600. The concentrate (30 g. equivalent to 10 g. of mixed vitamins A_1 and A_2) was dissolved in light petroleum (8 l.) and oxidized over MnO_2 (7 g.) for 4 days in darkness with occasional stirring. (The amount of MnO_2 was rather small as it was not desired to convert all the vitamin A_1 to retinene$_1$. Vitamin A_1 preponderates over vitamin A_2 and oxidation causes the 693 mμ. colour-test band of the latter to disappear first; the MnO_2 is then removed by filtration.)

The filtrate was reduced to small bulk, divided into three equal portions which were separately chromatographed on 8% watered alumina (200 g. in each column). Seven zones were seen on development with light petroleum; 2 and 3 (from top) contained most of the retinene$_2$ and 4 and 5 contained increasing amounts of retinene$_1$. The zones were separated by continuous percolation. All fractions enriched in retinene$_2$ were combined and rechromatographed on 10% watered alumina. The whole process was repeated once more and a final chromatography on 11% watered alumina gave 0·75 g. of retinene$_2$ free from retinene$_1$. This material crystallized at low temperature from light petroleum. The melting point (62°) rose with successive recrystallizations (5% solution in light petroleum) to 77–78°. After each crystallization about one-third of the solute is recovered as red crystals about 0·5 mm. long. The spectroscopic properties are shown in Fig. 1 and Table 1.

The preparation of retinene$_2$ is tedious but entirely reproducible given sufficient quantities of a suitable liver oil.

Analysis of retinene$_2$. The pure material is very readily oxidized on exposure to air. This tends to make values for C and H low; the substance is also rather difficult to burn at the ordinary combustion temperatures. Table 2 shows the results of many combustions.

Derivatives of retinene$_2$

(*a*) *Retinene$_2$-2:4-dinitrophenylhydrazone.* An ethanolic solution of 2:4-dinitrophenylhydrazine hydrochloride was added to an equivalent amount of retinene$_2$ in ethanol. On adding 1 drop of conc. HCl and warming gently, an immediate darkening was observed. A good crop of reddish brown needle-like crystals was deposited on cooling in the ice chest. The crystals were centrifuged and recrystallized five times from ethanol containing a little ether.

From retinene$_2$ from ling-cod and pike-liver oils it had m.p. 160–161°, $\lambda_{max.}$ 445 mμ. (EtOH).

From retinene$_2$ from Lake Nyasa oil it had m.p. 188–190°. $\lambda_{max.}$ 460 mμ. $E^{1\%}_{1\,cm.}$ 1240 (acetone), $\lambda_{max.}$ 460 mμ. $E^{1\%}_{1\,cm.}$ 1120 ($CHCl_3$) (see Fig. 2).

(*b*) *Retinene$_2$ semicarbazone.* To an aqueous-ethanolic solution of semicarbazide hydrochloride was added an ethanolic solution of retinene$_2$. The mixture was warmed in a current of inert gas, cooled and extracted with light petroleum-ether mixture (1:1) and the semicarbazone crystallized. It was recrystallized twice from a mixture of light petroleum and ether, when fine yellow crystals were obtained, m.p. 158°, $E^{1\%}_{1\,cm.}$ 1920 at 385 mμ. (EtOH), 1780 at 395 mμ. ($CHCl_3$).

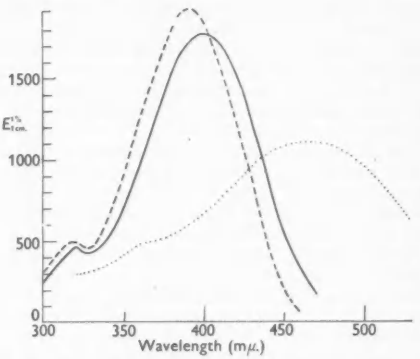

Fig. 2. Absorption spectrum of retinene$_2$-2:4-dinitrophenylhydrazone in chloroform, ·····; retinene$_2$ semicarbazone in ethanol, – – –; and chloroform, ——.

Antimony trichloride colour test

Fig. 3 shows the absorption spectrum of the greenish blue solution measured at definite time intervals after mixing retinene$_2$ (in $CHCl_3$) with the reagent (see Cama, Collins & Morton, 1951). Many solutions were examined at different wavelengths in different order so as to build up the com-

Table 2. *Analytical results on retinene$_2$ (m.p. 77–78°)*

(Analyses by Weiler and Strauss, Oxford, except those marked * which were carried out, with minimal exposure to air, in the Department of Organic Chemistry, University of Liverpool, by courtesy of Prof. A. Robertson, F.R.S.)

	C (%)	H (%)	O (%) (by difference)	Atomic ratio H/C	
Required for $C_{20}H_{26}O$	85·04	9·29	5·66	1·3	
Required for $C_{20}H_{28}O$	84·5	9·86	5·64	1·4	
Retinene$_2$	83·76	9·26	6·98	1·32	
	85·25*	9·28	5·47	1·31	Duplicates
	84·68*	9·34	5·98	1·32	
	83·8	9·0	7·2	1·29	One preparation
	81·14	8·9	9·9	1·31	
	83·5	8·9	7·6	1·28	
	83·36	9·0	7·64	1·29	
	84·0$_3$	9·3	6·67	1·33	One preparation
	84·40	9·7	5·9	1·37	
	84·7	9·6	5·7	1·36	
	84·2	9·5	6·3	1·35	
	85·6*	9·3	5·1	1·30	
	85·3*	9·38	5·32	1·32	
	83·1	8·77	8·13	1·27	
	83·3	8·95	7·75	1·29	

posite curves shown. The maximum slowly drifts from 730 to 740 mμ. as observed visually at the instant of mixing, to 705 mμ. where it remains for a long time.

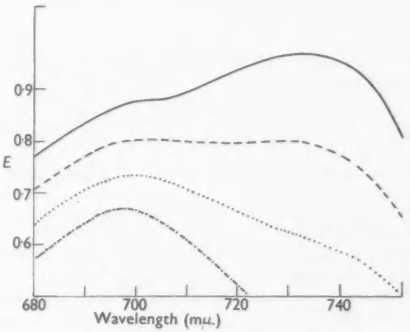

Fig. 3. Absorption curves on blue solution obtained by the interaction of retinene$_2$ and the $SbCl_3$ reagent; illustrating changes with time. ——, each wavelength extrapolated to zero time; – – –, E values at 4 min. after mixing; · · · · ·, E values at 8 min. after mixing; – · – · –, E values at 12 min. after mixing. (Absolute initial value in Table 1.)

DISCUSSION

If the term retinene$_2$ is restricted to the material obtained by Wald from freshwater-fish retinas, only qualitative descriptive properties are available, namely the ultraviolet absorption spectrum ($\lambda_{max.}$ 405 mμ. in chloroform) and a colour-test maximum at 705 mμ. The fact that our observed colour-test peak is at 730–740 mμ. would preclude identity of the pure material now obtained with Wald's retinene$_2$ were it not for the appearance of the 705 mμ. peak on standing. It is almost certain that Wald missed the initial phase which may, in fact, have been inhibited by congeners.

It is clear that our retinene$_2$ is an aldehyde derived from the vitamin A_2 of fish-liver oils. It cannot be doubted, in spite of the analytical difficulties revealed in Table 2, that the formula is not $C_{20}H_{28}O$ but $C_{20}H_{26}O$. The different analyses show how oxidation can occur (perhaps with formation of epoxides) or combustion can be incomplete. The structure (I) $C_{20}H_{28}O$ (related to lycopene) is definitely excluded. In any case, the ultraviolet absorption spectrum of such a compound should not differ from that of retinene$_1$. In fact the curve for retinene$_2$ is appreciably displaced in the direction of longer wavelengths (389 mμ. → 407 mμ. for the peaks in chloroform); this means that retinene$_2$ must have one additional conjugated double bond as compared with retinene$_1$. Further aspects of the structural problem are discussed in the following paper.

The apparent discrepancy over the melting point of the 2:4-dinitrophenylhydrazone has been resolved as follows. Prof. E. R. H. Jones, F.R.S., has informed us that the melting point is 196–197° ($E^{1\%}_{1\,cm.}$ 462 mμ. 1001 in chloroform), but that on heating isomerization occurs to give m.p. 163–167° ($\lambda_{max.}$ 448 mμ. $E^{1\%}_{1\,cm.}$ 866). Our two preparations melted at 188–190° and 160–161° respectively and the spectra agreed closely with those obtained by the Manchester workers.

It is highly probable that natural vitamin A_2 is a mixture of *cis-trans* isomerides and that the crude retinene$_2$ will also be a mixture. This may account for the fact that one preparation showed a melting point of 61° not raised by recrystallization. All later preparations, however, melted sharply at 77–78° after purification, but it is noteworthy that relatively little change in spectrophotometric properties occurred as a result of recrystallization. It may well be that on standing the isomers tend to give an equilibrium mixture and that recrystallization merely accumulates the least soluble isomer.

SUMMARY

1. The liver of ling cod (*Ophiodon elongatus*) contains vitamin A_1 and vitamin A_2 in the ratio 1/8–1/10. The partially sterol-free unsaponifiable fraction in light petroleum left to stand over manganese dioxide gives a mixture of retinene$_1$ and retinene$_2$. Separation can be effected by chromatography on watered alumina.

2. The liver oil of pike (*Esox lucius*), though not a very rich source of vitamins A, contains more A_2 than A_1 and the unsaponifiable fraction after oxidation contains the two retinenes, which can be separated.

3. Liver oil obtained from mixed large freshwater fishes of Lake Nyasa contains up to 15 % of mixed vitamins A (A_2/A_1 about 1/3). The sterol-free unsaponifiable fraction yields mixed retinenes on oxidation by manganese dioxide.

(I)

4. One preparation of retinene$_2$ showed m.p. 61° (not raised by recrystallization) but numerous subsequent preparations melted sharply at 77–78°. Retinene$_2$ is an aldehyde ($C_{20}H_{26}O$, forming a 2:4-dinitrophenylhydrazone and semicarbazone. Structures requiring $C_{20}H_{28}O$ are excluded. The absorption curves in several solvents are recorded e.g. 386 mμ. for $\lambda_{max.}$ and $E^{1\%}_{1\,cm.}$ 1440 in *cyclo*hexane. The antimony trichloride colour test is greenish blue, $\lambda_{max.}$ 740 mμ. initially, fading to 705 mμ.

5. Retinene$_2$ as obtained above agrees in its properties with the material obtained from bleached freshwater-fish retinas.

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Studies in Vitamin A

20. SOME PROPERTIES OF RETINENE$_2$

By H. R. CAMA, P. D. DALVI, R. A. MORTON and M. K. SALAH
Department of Biochemistry, University of Liverpool

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In those species which make use of rhodopsin, vitamin A_1 and retinene$_1$ are the main chemical links to the chemistry of scotopic vision and up to the present no alternative to vitamin A_1 has been suggested as the key substance in photopic vision. The reactions of retinene$_1$ with amino compounds have already been described (Ball, Collins, Dalvi & Morton, 1949). The behaviour of retinene$_2$ needs to be compared with that of retinene$_1$ in all respects which seem to have relevance to vision. Special interest attaches to indicator yellow$_2$ observed by Wald (1937, 1938–9*a*, *b*) in bleached freshwater-fish retinas and in Granit's electrophysiologically recorded modulators (Granit, 1947).

EXPERIMENTAL

Reactions of retinene$_2$ with amino compounds

General procedure. (*a*) A solution (4×10^{-5} M) of crystalline retinene$_2$ (Cama *et al.* 1952) in ethanol was prepared ($E_{1\,cm.}$ about 1·5 at 390–400 mμ.). To the solution (2 ml.) was added an aqueous solution (2 ml.) of an amino compound. In some cases the latter solution was nearly saturated but the concentration was always 10^{-3} M. To the mixture was added 0·1 N-NaOH (2 ml.) and the solution was left to stand at room temperature in the dark for 15–30 min. The absorption spectrum was then measured. The solution was then acidified (1 drop conc. HCl) and the absorption spectrum redetermined. For the alkaline solutions $\lambda_{max.}$ occurred at 380–400 mμ. and for the acidified solutions at 460–465 mμ.

(*b*) Amino compounds in which the —NH_2 group is attached directly to an aromatic ring react directly with retinene$_2$ to exhibit $\lambda_{max.}$ 530–560 mμ. on acidification. It is not necessary to add alkali first. Typical absorption curves are shown in Figs. 1 and 2 and values of $\lambda_{max.}$ are given in Table 1.

Interaction of retinene$_2$ and concentrated acids

(*a*) A solution of retinene$_2$ in $CHCl_3$ was cooled to 0° and mixed with an equal volume of conc. H_2SO_4 cooled to 0°. The $CHCl_3$ solution became green ($\lambda_{max.}$ 735 and 705 mμ.) and the acid became red ($\lambda_{max.}$ 570, 525, 470 mμ.). Retinene$_1$ tested similarly gave for the $CHCl_3$ solution $\lambda_{max.}$ 664 mμ. and for the acid solution 570, 525 and 470 mμ.

(*b*) A solution of retinene$_2$ in light petroleum was added to ice-cold syrupy phosphoric acid. The solution became blue

but the colour faded quickly especially in light. Maxima were initially recorded at 600, 570, 505 and 470 mμ., after 2 hr. at 590–600, 560, 505 and 470 mμ.; the two latter bands steadily gained intensity.

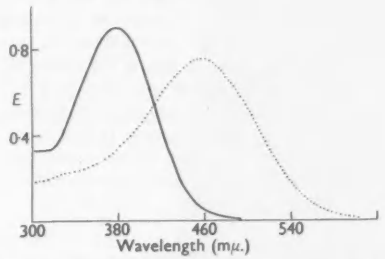

Fig. 1. Absorption spectrum of retinene$_2$ plus methylamine (in excess), solution slightly alkaline, ——; subsequently acidified, ·····.

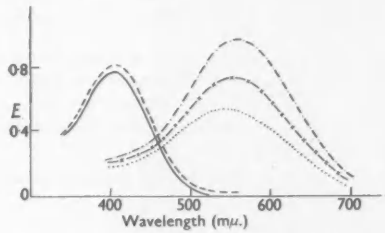

Fig. 2. Absorption spectrum of retinene$_2$ in aqueous ethanol plus *p*-aminobenzoic acid (in excess), ——; solution made alkaline, – – –; original solution made acid, –·–·–; acidified solution exposed to diffused light (2 hr.), ×—×; acidified solution exposed to diffused light (4 hr.), ·····.

DISCUSSION

Pure retinene$_2$ forms indicator yellow$_2$ analogues in exactly the same way as retinene$_1$ forms analogues of indicator yellow (Collins & Morton, 1950; Ball, Collins, Morton & Stubbs, 1948). The immediate reaction is probably

$$RCHO + H_2NX \rightarrow RCH{=}NX + H_2O,$$

and the change in $\lambda_{max.}$ amounts to no more than a slight displacement. Acidification results in a marked shift which chromophorically requires an ionized form presumably involving conjugation between two C_{20} units.

The shift from 465 to 520–560 mμ. suggests that compounds of the type RCH=NAr in an acid medium produce ionized forms with additional conjugation of unsaturated centres.

It is tempting to argue that in rhodopsin and porphyropsin the retinenes are combined with $-C_6H_4.NH_2$ groups but that would be too simple a view. All that can be said with certainty is that the present results permit the difference between rhodopsin ($\lambda_{max.}$ 500 mμ.) and porphyropsin ($\lambda_{max.}$ 522 mμ.) to be ascribed to the presence of the second double bond in the ring for retinene$_2$ and vitamin A$_2$. This is also sufficient to account for the difference between indicator yellow, and indicator yellow$_2$. A spectroscopic comparison of the two series is given in Table 2.

Table 1. *Wavelengths of maximum absorption of solutions obtained by mixing retinene$_2$ and amino compounds in aqueous ethanol*

Amino compound	$\lambda_{max.}$ (mμ.) (in alkali)	$\lambda_{max.}$ (mμ.) (after acidification)
(*a*) Alkylamino compounds		
Methylamine	380	460
Dimethylamine	400–405	400–405
Benzylamine	380	465
Glycine	380	465
β-Alanine	380	465
Serine	380	460
Tyrosine	380	460
Casein	380	Orange-yellow ppt.
Gelatin	380	460
(*b*) Arylamino compounds		
	As mixed (no added alkali)	Acidified
Aniline	400	535
Methylaniline	400	535
1-Naphthylamine	*	535
2-Naphthylamine	*	545
Benzylaniline	400	515
o-Toluidine	390	520
p-Toluidine	400	530
o-Aminophenol	405	520
p-Aminobenzoic acid	400	550
Anthranilic acid	*	560
p-Aminobenzenesulphonamide	400	560

* Not measured.

Table 2. *Spectroscopic comparison of the vitamin A$_1$ and A$_2$ systems in relation to vision*

	Vitamin A$_1$ $\lambda_{max.}$ (mμ.)	Vitamin A$_2$ $\lambda_{max.}$ (mμ.)
Vitamin (alcohol)	325	351, 286
$SbCl_3$ colour test	620	693
Retinene	370	385
$SbCl_3$ colour test	664	730–705
Rhodopsin	500	
Porphyropsin		525
Indicator yellow	365, 440	380, 465
Indicator yellow analogues	365, 440	380, 465
Retinene-*p*-aminobenzoic acid product	530	550
Retinene in conc. H_2SO_4	450, 525, 570, 664	470, 525, 570

The significance of the colour tests with antimony trichloride and strong acids remains somewhat uncertain. The 620 and 693 mμ. bands of vitamins A$_1$

and A_2 agree so closely with those of anhydrovitamins A_1 and A_2 as to make it clear that dehydration must here precede formation of the blue 'ionized' form. The 664 mμ. and 735 (705 mμ.) bands of the retinenes belong to ionized forms which can only be formulated speculatively; in any case there is no electrophysiological or chemical evidence to connect them unequivocally with known aspects of visual chemistry. The 'strong acid' peaks in the vitamin A series are plausibly analogous to Granit's photopic modulators. The results on retinene$_2$ neither add to, nor detract from, such plausibility. If, however, the existence of labile ions is held to show that vitamin A_1 is sufficiently versatile to be responsible for photopic vision, a rather important hint is provided by retinene$_2$. The absorption peaks of retinene$_2$ in sulphuric acid occur at nearly the same wavelengths as those of retinene$_1$. If the positions of the photopic modulators were found in the eyes of freshwater fish (or other 'porphyropsin' eyes) to be shifted as compared with those of mammalian or sea-fish eyes, the idea that these coloured ions possess some fundamental characteristics in common with the modulators would lose in force. The agreement between the wavelengths of absorption peaks of the two retinenes in concentrated sulphuric acid is unexpected. Unfortunately, it seems doubtful whether the electrophysiological methods can be made selective enough to provide a firm basis for fine distinctions in the positions of modulators.

To counterbalance the above it must be said that the first requirement in postulating a common key substance for scotopic and photopic vision (i.e. one in which vitamin A_1 or A_2 is equally important to both types of sensitivity) is that it should reproduce the Purkinje shift (500–565 mμ. in vitamin A_1 eyes). The necessary possibilities are provided by the grouping ACH=NR in which ACHO = retinene$_1$ or retinene$_2$, RNH_2 = amino group linked with aliphatic or aromatic residues. Chromophorically A and R could be reversed. Further investigation is, of course, necessary.

SUMMARY

1. Retinene$_2$ with aliphatic amines forms spectroscopic analogues of acid and alkaline indicator yellow$_2$. With aromatic amines it forms compounds which after acidification exhibit a new broad band with $\lambda_{max.}$ 560 mμ.

2. Retinene$_2$ reacts with concentrated sulphuric acid to give coloured unstable products with absorption peaks near 570, 525 and 470 mμ.

3. The bearing of these results on the chemistry of vision is discussed briefly.

We are indebted to the Medical Research Council for financial assistance.

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Studies in Vitamin A

21. RETINENE$_2$ AND VITAMIN A_2

By H. R. CAMA, P. D. DALVI, R. A. MORTON and M. K. SALAH
Department of Biochemistry, University of Liverpool

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A preceding paper (Cama *et al.* 1952) records the preparation of crystalline retinene$_2$ from various sources. When retinene$_1$ is fed to rats it is reduced in the lining of the intestine to vitamin A_1 and the esterified vitamin is then carried to the liver and stored (Glover, Goodwin & Morton, 1948). Retinene$_1$ may be reduced to vitamin A_1 either by the Ponndorf method or by the use of lithium aluminium hydride ($LiAlH_4$).

The present paper records the similar conversion *in vivo* and *in vitro* of retinene$_2$ to vitamin A_2. The properties of vitamin A_2 are discussed with particular reference to the rather vexed question of chemical constitution.

EXPERIMENTAL

Feeding experiments with retinene$_2$

Formation of vitamin A$_2$. Groups of young rats (weaned and accustomed to a normal diet) were transferred to a vitamin A- and carotenoid-free diet known from previous experience to result in vitamin A deficiency. When a group began to lose weight, one rat was killed and the liver examined for vitamin A. In all cases the liver reserve was found to be very low and it was usually negligible.

Rats from such groups, when losing weight, were given a dose of retinene$_2$ in arachis oil (0·5–1 ml.); 24 hr. later they were anaesthetized and killed. The livers were then examined for vitamin A$_2$.

Example. Retinene$_2$, m.p. 61° (Cama *et al.* 1952), was dissolved in arachis oil (8 mg./ml.) and a dose of 0·5 ml. given to each rat. The liver from an animal killed 24 hr. later was ground with silver sand and Na_2SO_4 and extracted with redistilled diethyl ether. The ether was removed in a current of N_2 and part of the residue was dissolved in $CHCl_3$. When tested by means of the $SbCl_3$ reagent a greenish blue colour was obtained ($\lambda_{max.}$ 693 mμ. well defined; no 620 mμ. band to be seen; $E_{693\ m\mu.}/E_{620\ m\mu.} = 2{\cdot}7$). When a solution of the liver lipid in *cyclo*hexane was examined for ultraviolet absorption, maxima at 347·5, 287 and 275 mμ. were recorded. With similar preparations chromatographic separations on bone meal showed that the liver lipids contained both esterified and free vitamin A$_2$.

When retinene$_2$ of m.p. 77–78° (Cama *et al.* 1952) was used the results were similar (except that the minor absorption peak at 275 mμ. was not always seen). Vitamin A$_2$ recovered from the livers 18–24 hr. after dosage with retinene$_2$ amounted to about 25% of that theoretically possible. With retinene$_1$ 'yields' of vitamin A$_1$ were nearer 50%.

Using an uncrystallized concentrate of retinene$_2$ (free from retinene$_1$ but by spectrophotometric assay containing 75% retinene$_2$) liver storage of vitamin A$_2$ was roughly in proportion to the dose.

Rather large single doses of retinene$_2$ (130 μg.) and vitamin A$_2$ (120 μg.) promptly cured early xerophthalmia and restored growth, but quantitative biological assays were not carried out.

Replacement of vitamin A by vitamin A$_2$. Three albino rats 6 weeks old and weighing about 90 g. each were placed on a vitamin A-free diet. All the animals grew slowly, but from the 13th to the 15th week weights were stationary or falling. Each rat was then given retinene$_2$ (about 33 μg.) in arachis oil twice a week orally by means of a syringe. Growth was promptly resumed and continued slowly for the next 3 months. Dosage was discontinued from the 22nd to the 24th week and then resumed, but there was no break in growth. At the age of 36 weeks the rats were kept in the dark for 18 hr., anaesthetized in a red light and killed by drawing blood (cardiac puncture) into a syringe previously moistened with potassium oxalate solution. The livers and kidneys of each rat were tested for vitamins A and the bulked blood was similarly assayed. Results are sumarized in Table 1. The six retinas were combined and it was not apparent on testing for rhodopsin that any detectable replacement of rhodopsin by porphyropsin had occurred.

Ponndorf reduction of retinene$_2$

Crystalline retinene$_2$ (5 mg., m.p. 77–78°, $E^{1\%}_{1\ cm.}$ 386 mμ., 1450) in *iso*propanol was refluxed with aluminium *iso*propoxide (0·3 g.) for 18 hr. on the water bath. The refluxing was twice interrupted after 5 hr. and the mixture kept at 0° overnight and at each stage the product was tested. When the colour-test maximum at 693 mμ. was shown strongly, the heating was discontinued. After cooling, the mixture was treated with KOH to decompose the alkoxides and extracted with light petroleum. After washing, the solvent was removed under suction. The dry residue was dissolved in light petroleum and poured onto a column (2 × 1·75 cm.) of weakened alumina (10% water, w/w). On development with light petroleum, unchanged retinene$_2$ (about 0·4 mg.) quickly passed through the column leaving a yellow adsorbed zone. Elution with ethanol gave vitamin A$_2$, free from substances showing interfering absorption. The yield of vitamin A$_2$ was approximately 50% of the retinene$_2$ which reacted. The spectrum of vitamin A$_2$ obtained in this way shows only one definite maximum in the region 270–295 mμ. and the curve with $\lambda_{max.}$ 351 and 286 mμ. agrees very well with that of Shantz (1948).

In another experiment 35 mg. of crystalline retinene$_2$ were subjected to reduction (Ponndorf) and the product worked up as before. After two chromatographic adsorptions an apparently homogeneous fraction containing about 15 mg. vitamin A$_2$ was obtained. This was dissolved in light petroleum (2 ml.) and left at −70° to crystallize. After 2 months, supernatant liquor was decanted from a small amount of solid. A little fresh solvent was added but the vitamin A$_2$ would not crystallize. The solvent was then removed and the residue weighed carefully (about 3 mg.). The following figures were obtained: $E^{1\%}_{1\ cm.}$ 351 mμ., 1490 (light petroleum) and $E^{1\%}_{1\ cm.}$ 693 mμ. (colour test), 4000 (see Figs. 1 and 2). Although high accuracy is not claimed we regard these measurements as trustworthy within a few per cent.

Yields of vitamin A$_2$ from retinene$_2$ by this process are not, however, satisfactory.

Table 1. *Vitamins A$_1$ and A$_2$ in rats given retinene$_2$ (0·33 μg. twice weekly) for 3 months*

Rat no.	Weight (g.)			Vitamin A$_1$ and A$_2$ content (i.u./g.)				Volume (ml.)		Vitamins A$_1$ and A$_2$ in i.u. (1 ml. serum)*	
				Liver		Kidney					
	Body	Liver	Kidney	A$_1$	A$_2$	A$_1$	A$_2$	Blood	Serum	A$_1$	A$_2$
1	250	10·9	2·3	115	52	8·6	3·6	9·5	4·6		
2	218	7·2	1·8	4	5	14·0	8·5	7·5	3·3	1·83	0·95
3	275	9·7	2·3	10	15	13·7	9·8	9·5	4·4		

* Combined sample.

Reduction of retinene$_2$ by $LiAlH_4$

Lithium aluminium hydride (200 mg.) was finely ground and dissolved in anhydrous diethyl ether (20 ml.). Crystalline retinene$_2$ (50 mg.) was also dissolved in dry ether (50 ml.). The reagent was added slowly with stirring at 0°. The solution quickly became pale yellow and then almost colourless (4–5 min., protected against strong light). (If the solution is left for a longer time it turns violet and the yield of vitamin A_2 is much reduced.) The excess $LiAlH_4$ was decomposed by dropwise addition of water, the flask being cooled by means of ice water. The mixture was extracted with redistilled ether and the ethereal layer washed and then dried (Na_2SO_4). The solvent was removed under reduced pressure. The residue (30 mg., $E^{1\%}_{1\,cm.}$ 351 mμ., 970) was mainly vitamin A_2 (693 mμ. peak in colour test). This was chromatographed on alumina weakened by addition of water (10%, w/w). A very small fraction showing maxima at 350 and 285 mμ. with an inflexion at 275 mμ. was carried through by light petroleum (neovitamin A_2?). This was followed by a fraction showing maxima at 320 and 345–350 mμ. Development with light petroleum-diethyl ether (95/5, v/v) gave a vitamin A_2 fraction (15 mg.). Doubling the proportion of ether resulted in elution of a small amount of oxidized material and anhydrovitamin A_2. Other oxidation products were eluted by pure ethanol. Rechromatographing the vitamin A_2 fraction gave a fraction eluted by

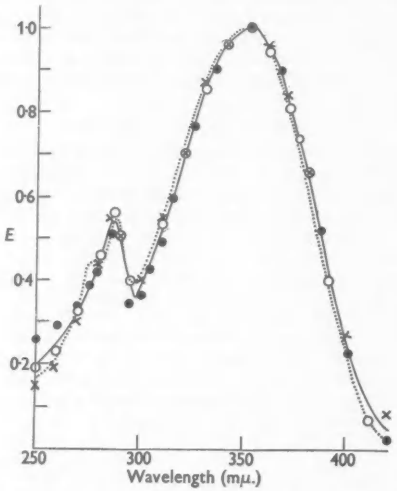


Fig. 1. Absorption spectrum of vitamin A_2 in ethanol, ——; preparations obtained by reduction of retinene$_2$ *in vivo*, ●; *in vitro* (Ponndorf), ○; ($LiAlH_4$), · · · · ·; vitamin A_2 (from Shantz, 1948), ×.

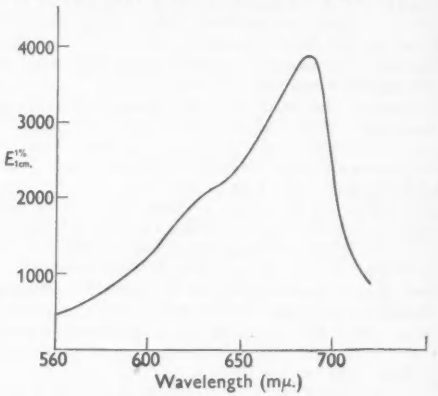


Fig. 2. Absorption curve on the blue solution obtained by the interaction of vitamin A_2 and the $SbCl_3$ reagent.

Table 2. *Spectroscopic properties of vitamin* A_2

Solvent	$\lambda_{max.}$ (mμ.)	$E^{1\%}_{1\,cm.}$	Inflexion (mμ.)	$E_{287\,m\mu}/E_{350\,m\mu}$
Ethanol*	352	1460	None	0·55
	287	820	None	—
Ethanol†	352	1330	None	0·51
	288	678	None	—
Ethanol‡	351	1410	277	$0{\cdot}54_5$
	286	698	—	—
Light petroleum‡	348	1390	277	0·54
	286	750	—	—
*cyclo*Hexane‡	351·5	1320	275	$0{\cdot}55_5$
	287	733	—	—
*iso*Propanol‡	351	1370	277	0·55
	285	753	—	—
Chloroform‡	356·5	1280	282	0·55
	291·5	704	—	—
$SbCl_3$ colour test*	693	4100	—	—
$SbCl_3$ colour test†	693	3700	—	—
$SbCl_3$ colour test‡	693	3870	—	—

* Shantz (1948). † Farrar, Hamlet, Henbest & Jones (1951). ‡ Present work.

5% ether-light petroleum, which in *cyclo*hexane showed the 350 and 286 mμ. maxima and a barely perceptible step-out at 275 mμ. (see Fig. 1).

The properties of vitamin A$_2$ are shown in Table 2.

Preparation of anhydrovitamin A$_2$

To about 8 mg. of vitamin A$_2$ were added 15 ml. approx. 0·03 N-ethanolic HCl (anhydrous). The solution was left to stand at room temperature in the dark for 15 min. The greenish solution was neutralized, extracted with redistilled ether and finally chromatographed.

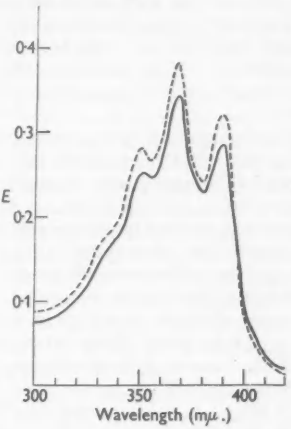


Fig. 3. Absorption spectrum of anhydrovitamin A$_2$ in ethanol, ——; and in light petroleum, - - - - -. (Absolute values not certain.)

The absorption spectrum (Fig. 3) is entirely characteristic, but the product differs from anhydrovitamin A in giving a 693 mμ. band in the colour test as compared with 620 mμ.

DISCUSSION

There is little hope of isolating sufficient retinene$_2$ from retinas for more than spectroscopic characterization. Proof that Wald's retinene$_2$ is the aldehyde of vitamin A$_2$ therefore rests on cumulative evidence which must include precise information about vitamin A$_2$ itself.

Karrer & Bretscher (1942) and Karrer, Bretscher & Geiger (1943) prepared from pike-liver oil a vitamin A$_2$ concentrate with $\lambda_{max.}$ 345 mμ. and $E^{1\%}_{1\,cm.}$ 1450. This was probably impure. Shantz (1948) observed $\lambda_{max.}$ 352 mμ. $E^{1\%}_{1\,cm.}$ 1460 (ethanol) and $\lambda_{max.}$ 287 mμ. $E^{1\%}_{1\,cm.}$ 820, with $\lambda_{max.}$ 693 mμ. $E^{1\%}_{1\,cm.}$ 4100 in the colour test. Although vitamin A$_2$ has itself never yet been obtained crystalline, its *p*-phenylazobenzoate (m.p. 76–77°) has been crystallized. On the basis of the open chain (ψ-ionone) formula advocated earlier (Karrer, Geiger & Bretscher, 1941), the empirical formula would be $C_{33}H_{38}O_2N_2$. (Required C, 80·1; H, 7·7; N, 5·7%. Found: C, 80·1; H, 7·8; N, 5·9%.) This agreement implies that vitamin A$_2$ is isomeric with vitamin A$_1$ ($C_{20}H_{30}O$). Anhydrovitamin A$_2$ (m.p. 89·5°) with seven double bonds apparently corresponded with $C_{20}H_{28}$ (open-chain formula). (Required C, 89·5; H, 10·5%. Found: C, 89·2; H, 10·7%.) Against this, if retinene$_2$ is $C_{20}H_{28}O$ (Cama *et al.* 1952), it is difficult to consider any formula but $C_{20}H_{28}O$ for vitamin A$_2$, and $C_{20}H_{26}$ for anhydrovitamin A$_2$.

Karrer & Schneider (1950) re-investigated vitamin A$_2$ *p*-phenylazobenzoate and raised the melting point to 94–95° but lacked material for further recrystallization. The rise in melting point from 76–77° (Shantz) to 94–95° may mean that the preparations were isomeric (cf. Farrer, Hamlet, Henbest & Jones, 1951). $C_{33}H_{38}O_2N_2$ (mol.wt. 494·3) requires C, 80·1; H, 7·7%; whereas $C_{33}H_{36}O_2N_2$ (mol.wt. 492·3) requires C, 80·4; H, 7·3%. (Found: C, 80; H, 7·2%.) As the analyses were not quite decisive, Karrer & Schneider (1950) treated vitamin A$_2$ (recovered from the *p*-phenylazobenzoate) with ozone and failed to obtain acetone, although they had no difficulty in detecting it in the ozonization products of ψ-ionone and ψ-ionylidene-ethanol. This result seems to exclude the open-chain formula $C_{20}H_{30}O$ for vitamin A$_2$ and, by implication, $C_{20}H_{28}O$ for retinene$_2$.

Farrar *et al.* (1951) have synthesized vitamin A$_2$ via 3-dehydrovitamin A$_1$ acid, and their 3-dehydrovitamin A$_1$ cannot be distinguished from vitamin A$_2$. Moreover, their retinene$_2$ prepared by the method of Ball, Goodwin & Morton (1948) (MnO_2 oxidation) is identical with our crystalline retinene$_2$. The spectroscopic characterization will be discussed later.

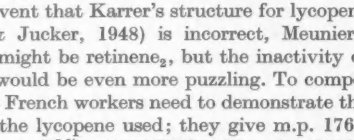
Now vitamin A$_2$ is biologically active. It is generally agreed that it can (at least partially) act as a substitute for vitamin A$_1$ in rats and there is no reason to doubt its biological activity in all species where it occurs naturally. Shantz & Brinkman (1950) have shown that, for rats, spectroscopically pure vitamin A$_2$ possesses about 40% of the potency of an equal weight of vitamin A$_1$. The biogenesis of vitamin A$_2$ remains obscure, but from the work of Morton & Creed (1939) perch can use β-carotene as a vitamin A$_2$ precursor. It is not clear whether vitamin A$_1$ must be made first, i.e. whether or not dehydrogenation precedes cleavage of the C_{40} compound. All the available evidence indicates that unless a carotenoid contains one intact β-ionone ring it cannot act as a provitamin A$_1$. This would, of course, exclude lycopene, with an open-chain structure.

The interesting work of Meunier, Jouanneteau & Zwingelstein (1950) and Meunier (1951) shows that manganese dioxide can be used effectively to split the β-carotene molecule and yield retinene$_1$. This

finding is in harmony with other work (Goss & McFarlane, 1947; Wendler, Rosenblum & Tishler, 1950) and serves to emphasize the versatility of manganese dioxide as an oxidizing agent simulating the action of dehydrogenases *in vivo*.

The next step in the work of Meunier (1951), however, requires very careful scrutiny. Lycopene undergoes fission with manganese dioxide in a manner analogous to β-carotene to give an aldehyde said to be retinene$_2$ and to be reducible to vitamin A$_2$. Biological tests are described which indicate that the lycopene fission product is growth-promoting to rats on a vitamin A-deficient diet at a dose level of 6 μg./day. From these results it is argued that Karrer's original open-chain formula for vitamin A$_2$ is correct.

This work raises difficult questions. If it can be confirmed, the absence of biological activity for lycopene itself is difficult to understand. In the unlikely event that Karrer's structure for lycopene (Karrer & Jucker, 1948) is incorrect, Meunier's aldehyde might be retinene$_2$, but the inactivity of lycopene would be even more puzzling. To compel assent the French workers need to demonstrate the purity of the lycopene used; they give m.p. 176°, $\lambda_{max.}$ 485 mμ., $E^{1\%}_{1\,cm.}$ 1950 ($CHCl_3$) (Meunier, 1951), whereas Zechmeister, Le Rosen, Schroeder, Polgar & Pauling (1943) give m.p. 175°, $\lambda_{max.}$ 474 mμ., $E^{1\%}_{1\,cm.}$ 3480. If the $E^{1\%}_{1\,cm.}$ values found by Zechmeister *et al.* are correct the purity of the lycopene used by Meunier was only 66%, but the melting point argues against such a suggestion.



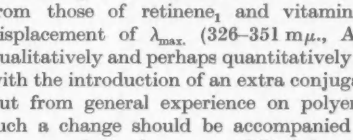
Vitamin A$_2$

Meunier also needs to demonstrate that his aldehyde is identical with retinene$_2$. As we have shown, $\lambda_{max.}$ in the colour test is 730–740 mμ. fading to 705 mμ. Meunier *et al.* record 715 mμ. The ultraviolet absorption should show $\lambda_{max.}$ 408 mμ. with an inflexion near 315 mμ. ($CHCl_3$). No information is given to suggest that this inflexion occurs in the aldehyde from lycopene.

At this stage it is necessary to discuss the spectroscopic properties of vitamin A$_2$. There is no doubt that the subsidiary absorption peak at 286 mμ. must be accepted as no less characteristic of vitamin A$_2$ than the main peak at 351 mμ. In fact, constancy of the ratio $E_{351\,m\mu}/E_{286\,m\mu}$ is a good criterion of purity (see Table 2). The absorption curve for the reduction product of the aldehyde derived from lycopene shows $\lambda_{max.}$ 352 mμ. in ethanol, but no mention is made of a 286 mμ. maximum (Meunier, 1951). The 693 mμ. absorption band in the colour test characteristic of vitamin A$_2$ also appears (690 mμ.) in Meunier's product. He does not, however, give E values, and although the aldehyde from lycopene is very like retinene$_2$, the evidence for identity falls considerably short of proof.

It cannot be doubted that the case in favour of the open-chain formula has not been made out. It is of course possible that the rat may be able to close a ring from the ψ-ionone part of the lycopene molecule, but this is not part of the argument advanced by Meunier although it could account for the biological activity. If so, however, the question inevitably recurs, why is the rat unable to use lycopene?

The spectroscopic properties of retinene$_2$ and vitamin A$_2$ could not, however, have been predicted from those of retinene$_1$ and vitamin A$_1$. The displacement of $\lambda_{max.}$ (326–351 mμ., A$_1 \rightarrow$ A$_2$) is qualitatively and perhaps quantitatively consistent with the introduction of an extra conjugated bond, but from general experience on polyene spectra such a change should be accompanied by a rise in $\epsilon_{max.}$ when electron mobility is unhindered. Actually $\epsilon_{max.}$ falls from about 50 000 to about 38 000. Both vitamin A$_2$ and retinene$_2$ have unexpectedly low $\epsilon_{max.}$ values. In the case of vitamin A$_2$ the fall in $\epsilon_{max.}$ is accompanied by the appearance of the subsidiary sharp absorption peak at 286 mμ.



Retinene$_2$

This means that the observed absorption curve is made up of three contributions: (*a*) that due to six conjugated double bonds ($\lambda_{max.}$ 351 mμ.); (*b*) that due to a proportion of the solute molecules in which less than six (perhaps two) conjugated double bonds give rise to the 286 mμ. band, i.e. due to a molecular 'partial'; (*c*) that due to the remaining molecular 'partial' with perhaps four conjugated double bonds. (For a discussion of 'bands of partial oscillation' see Lewis & Calvin (1933) and MacColl (1947).)

The ring structure alone might well give rise to the 286 mμ. band (cf. 7-dehydrocholesterol with peaks at 270, 281·5 and 293·5 mμ.) but, if so, a second band at 300–310 mμ. would certainly be expected for the other 'partial'. No such peak is observed in vitamin A$_2$, but it could be masked by

overlapping contributions from the (*a*) and (*b*) absorptions.

The displacement of $\lambda_{max.}$ in retinene$_2$ (vitamin A$_2$ → retinene$_2$; 351 → 385 mμ.) can be ascribed to the —CHO group conjugated to the main system, but the lack in retinene$_2$ of a sharp secondary peak to correspond with the 286 mμ. of vitamin A$_2$ is not easy to explain. There is a definite inflexion near 310 mμ., but neither the intensity nor the poor definition is consistent with the probability of 'partials' being the same as in vitamin A$_2$.

The small band near 275 mμ. shown by vitamin A$_2$ preparations is puzzling. It appears very regularly in certain freshwater fish liver oils and their unsaponifiable fractions rich in A$_2$. This is particularly true for Nile fishes (Salah, private communication). We have obtained some preparations (by Ponndorf reduction of retinene$_2$) in which the inflexion was not noticeable, but all our most recent reduction products ($LiAlH_4$) from purified retinene$_2$ show the inflexion even after careful chromatography of the product. On the other hand, the best preparations of Farrar *et al.* (1951) do not show the inflexion (private communication from Prof. E. R. H. Jones) nor do those of Shantz (1948). At present it is difficult to assert that the inflexion is due to an impurity rather than a vitamin A$_2$ isomer and the matter must be left open.

The vitamin A$_2$ obtained by reduction of retinene$_2$ shows the $SbCl_3$ colour-test peak at 693 mμ. ($E^{1\%}_{1\,cm.}$ about 4000) with a weak inflexion near 640 mμ. (Fig. 2). Most workers on vitamin A$_2$ have observed this inflexion, but it is possible that when vitamin A$_2$ is obtained as a single isomer it may be absent (cf. Shantz, 1948) just as the secondary colour-test selective absorption at 580 mμ. is not shown by the purest *all-trans* vitamin A$_1$ (Cama, Collins & Morton, 1951).

The vitamin A$_2$ obtained in the present work forms anhydrovitamin A$_2$ smoothly and the product shows the expected spectrum (Fig. 3). The fact that anhydrovitamins A$_1$ and A$_2$ show identical absorption maxima, but differ in respect of their colour-test maxima, is confirmed. Plausible structures have been advanced by various groups of workers, but objections can be raised to all of them and in our view new evidence is needed before the matter can be settled.

SUMMARY

1. When retinene$_2$ is fed to avitaminotic rats receiving a diet free from carotenoids or vitamin A, it is converted to vitamin A$_2$ which can be found in the liver within 24 hr. The recovered vitamin A$_2$ shows an absorption spectrum agreeing very well with that of the purest preparations.

2. Vitamin A$_2$ is moderately well stored by rats but is not a wholly satisfactory substitute for vitamin A$_1$.

3. Reduction of retinene$_2$ by the Ponndorf and lithium aluminium hydride methods results in vitamin A$_2$, the product being indistinguishable from the purest natural vitamin A$_2$ preparations.

4. The $SbCl_3$ colour reaction of vitamin A$_2$ has been studied in detail.

5. The evidence is very strong that vitamin A$_2$ is 3-dehydrovitamin A$_1$.

We are indebted to the Medical Research Council for financial assistance.

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Studies in Vitamin A

22. OPPENAUER OXIDATION OF VITAMIN A_1

By H. R. CAMA, A. C. FIELD, J. GLOVER, R. A. MORTON and M. K. SALAH
Department of Biochemistry, The University of Liverpool

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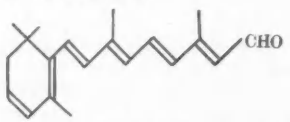
The merit of the Oppenauer oxidation (Oppenauer, 1937; reviewed by Djerassi, 1951) using aluminium alkoxides and a hydrogen acceptor, is that unsaturated alcohols can be converted to aldehydes and ketones without affecting the ethylenic linkages. Applied to vitamin A alcohol it ought to yield retinene$_1$ (vitamin A_1 aldehyde) as the primary product. Hawkins & Hunter (1944*a*), using aluminium *iso*propoxide and acetaldehyde as hydrogen acceptor, have in fact obtained retinene$_1$. There is, however, a marked tendency for secondary reactions to occur and so to diminish the yield of the initial dehydrogenated product. Haworth, Heilbron Jones, Morrison & Polya (1939) varied the conditions, and in particular used aluminium *tert.*-butoxide and diethyl ketone as hydrogen acceptor. They did not obtain retinene, but found instead that a crystalline oxime (isolated in low yield from the reaction mixture) corresponded with an 'aldehyde' $C_{20}H_{26}O$. At that time there was no suspicion that the retinenes were the aldehydes of vitamins A_1 and A_2 and for a time the observation attracted no special interest.

The tentative structure



assigned to the C_{20} aldehyde implied dehydrogenation in the β-ionone ring as well as at the primary alcohol grouping. This structure was later attributed to retinene$_2$ (Morton, Salah & Stubbs, 1947) largely on the basis of the properties of crystalline vitamin A_2 aldehyde prepared from natural vitamin A_2.

Synthetic retinene$_2$ has recently been prepared (Farrar, Hamlet, Henbest & Jones, 1951) and its properties agree very well with those of the retinene$_2$ prepared from vitamin A_2 of natural origin (Cama *et al.* 1952*b*).

The C_{20} aldehyde obtained by Haworth *et al.* (1939) was not fully characterized, except through the oxime, and it was clearly necessary to repeat the work, aiming at isolation and characterization of the aldehyde itself. This has proved to be a rather difficult matter because the Oppenauer product is always heterogeneous.

It will be shown that the $C_{20}H_{26}O$ preparation can be reproduced, and that the substance very closely resembles retinene$_2$ but is actually a totally distinct compound, the precise structure of which remains uncertain.

EXPERIMENTAL

Materials

Vitamin A concentrates. (1) Much of our earlier work was carried out on partially sterol-free unsaponifiable fractions from high-potency Portuguese tunny-liver oils. (2) Later, many experiments were made using similar fractions from ester concentrates. The unsaponifiable fractions, after crystallizing out much sterol, usually contained 50–55% vitamin A. (3) Finally, synthetic vitamin A acetate (Roche) was used after careful saponification. Some experiments were carried out on the acetate itself.

Reagents. Aluminium *tert.*-butoxide was prepared by the method of Wayne & Adkins (1941). Aluminium *iso*propoxide was prepared according to Wilds (1944). Benzene was dried over Na wire and redistilled before use. Diethyl ketone (commercially pure) was dried over anhydrous $CuSO_4$, filtered and distilled.

Oppenauer reaction

The following experiment was typical. An all-glass refluxing apparatus was used and reactants were added in the following order: vitamin A concentrate (equivalent to 6 g. vitamin A), diethyl ketone (30 ml.), benzene (200 ml.) and aluminium *iso*propoxide (7·3 g.). The mixture was gently refluxed continuously (48 hr.). From the dark-red reaction mixture two small equal portions were taken and the aluminium alkoxides were decomposed in one by means of water and in the other by 10% H_2SO_4 (v/v). Light petroleum extracts from the two experiments could not be distinguished spectroscopically, and it was accepted that the acid had no deleterious effect. The main portion was then treated with excess of 10% H_2SO_4 (little emulsification occurs with acid) and light petroleum was added. After washing with $NaHCO_3$ solution and water the extract was dried (Na_2SO_4) and the solvent evaporated completely under reduced pressure. The residue was dissolved in light petroleum (50 ml.) and chromatographed on alumina (Peter Spence and Co., Grade O, weakened by addition of water, 10% w/w). The main fraction 2 (Table 1) was rechromatographed on alumina containing 8% water and a fraction (2*d*) was retained and rechromatographed over

alumina containing 11% water to give two distinct fractions, $2d_{3-9}$ and $2d_{10-15}$, which were separately crystallized at low temperature (−60°) from light petroleum. After three crystallizations the two products were analysed.

From Table 2 it will be seen that the less strongly adsorbed compound is probably $C_{20}H_{26}O$. It gives an oxime of m.p. 175–177° agreeing with that recorded by Haworth *et al.* (1939), Hawkins & Hunter (1944*b*) and Hunter (1950).

Table 1. *Properties of fractions obtained by chromatography*

Fraction	Wt. (g.)	Absorption maxima (mμ.)	$SbCl_3$ colour-test maxima (mμ.)
Stage 1. Alumina containing 10% water			
1 (yellow)	0·19	355, 370, 390, 415	715, 620
2 (dark red)	8·0	375, 390	715–720
3 (yellow)	0·37	330	710 weak, 620 strong
4 (yellow)	0·13	330	620
5* (red)	1·7	310	620
Stage 2. Alumina containing 8% water			
2*a* (yellow)	—	270, 350, 370, 390	620
2*b* (yellow)	—	270, 350, 370, 390	620
2*c* (red)	—	330, 350, 365, 390	710
2*d* (red)	—	385	715
2*e** (red)	—	330	640
Stage 3. Alumina containing 11% water			
$2d_{1-2}$	—	380	695–700 $E^{1\%}_{1\,cm.}$ 1170
$2d_{3-4}$	—	385	~685
$2d_{5-9}$	—	385	720 $E^{1\%}_{1\,cm.}$ 1085 (640 weak)
$2d_{10-15}$	—	385	720 $E^{1\%}_{1\,cm.}$ 1050
$2d_{16}$*	—	375–385	640–715

* Eluted by means of ether.

Table 2. *Analytical results and spectroscopic properties of* $C_{20}H_{26}O$ *compound obtained by Oppenauer oxidation of vitamin A* (*m.p.* 79–80°)

(*a*)

	C (%)	H (%)	O (%)	H/C (atoms)
Found	84·93	9·38	5·69	1·31
$C_{20}H_{26}O$ requires	85·04	9·29	5·66	1·3
$C_{20}H_{28}O$ requires	84·5	9·86	5·64	1·4

(*b*)

Solvent	$\lambda_{max.}$ (mμ.)	$E^{1\%}_{1\,cm.}$	Inflexion (mμ.)
Light petroleum	386	1690	270
*cyclo*Hexane	390	1740	270
Ethanol	395	1450	280
Chloroform	406	1480	280
$SbCl_3$ colour test	720	—	—

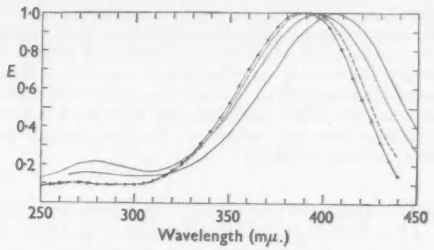


Fig. 1. Absorption spectrum of $C_{20}H_{26}O$ compound in light petroleum, •—•; *cyclo*hexane, – – –; ethanol, · · · ·; and chloroform, ——.

Table 3. *Properties of the oxime of the* $C_{20}H_{26}O$ *compound* (*m.p.* 175–177°)

(i)

	C (%)	H (%)	N (%)
$C_{20}H_{27}ON$ requires	80·8	9·2	4·7
$C_{20}H_{29}ON$ requires	80·2	9·8	4·7
Found	81·14	9·57	4·65 (*a*)
	80·6	9·8	4·6 (*b*)
	80·5	10·0	4·5 (*b*)
	80·1	9·55	4·5 (*c*)
	80·2	9·75	4·8 (*c*)

(ii)

	(*a*)	(*c*)
$\lambda_{max.}$ (light petroleum)	375 mμ.	380 mμ.
$E^{1\%}_{1\,cm.}$	1890	1940*

(*a*) Present work.
(*b*) Haworth *et al.* (1939).
(*c*) Hawkins & Hunter (1944*b*).

* Misprint in original paper.

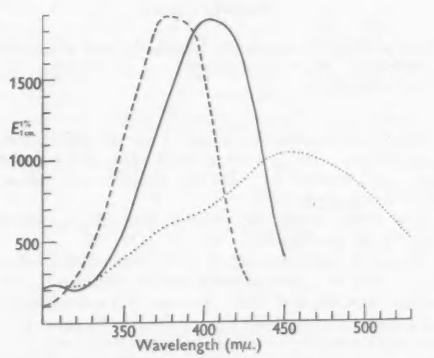


Fig. 2. Absorption spectrum of $C_{20}H_{26}O$-2:4-dinitrophenylhydrazone in chloroform, · · · · ·; $C_{20}H_{26}O$-semicarbazone in chloroform, ——; and $C_{20}H_{26}O$-oxime in light petroleum, – – –.

The $C_{20}H_{26}O$ compound (0·15 g.) was dissolved in ethanol, and hydroxylamine hydrochloride (0·1 g.) and sodium acetate (0·5 g.) were added in a small volume of water (5 or 6 drops). The mixture was refluxed (2 hr.) under N_2, cooled, poured into water (250 ml.) and left to stand at 0° overnight. The flocculent yellow precipitate was filtered off and recrystallized from light petroleum. The properties of the oxime are listed in Table 3.

Table 4. *Analytical results and spectroscopic properties of C_{25} ketone obtained by Oppenauer oxidation of vitamin A in presence of diethyl ketone* (*m.p.* 76–78°)

(*a*)

	C (%)	H (%)	O (%)
Found (present work)	85·27	9·66	5·07
(Jones, 1951)	85·6	10·6	3·8
$C_{25}H_{36}O$ requires	85·15	10·3	4·65
$C_{25}H_{34}O$ requires	85·65	9·7	4·65

(*b*)

Solvent	$\lambda_{max.}$ (mμ.)	$E^{1\%}_{1\,cm.}$	Inflexion (mμ.)
Light petroleum	390	1354	270
*cyclo*Hexane	390	1415	275
Ethanol	400	1271	275
Chloroform	408	1223	280
$SbCl_3$ colour test	720, 640	—	—

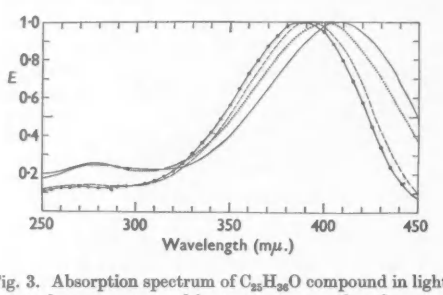


Fig. 3. Absorption spectrum of $C_{25}H_{36}O$ compound in light petroleum, •—•; *cyclo*hexane, – – –; ethanol, · · · ·; and chloroform, ——.

The 2:4-dinitrophenylhydrazone (m.p. 160–162°) showed $\lambda_{max.}$ 455 mμ., $E^{1\%}_{1\,cm.}$ 1050 ($CHCl_3$). The semicarbazone m.p. 152°, showed $\lambda_{max.}$ 390 mμ. (EtOH) and 400 mμ. ($CHCl_3$) with $E^{1\%}_{1\,cm.}$ 1860.

The second crystalline product (from $2d_{10-15}$) showed m.p. 76–78° (see Table 4).

By using aluminium *tert.*-butoxide (and diethyl ketone) the product is a mixture which contains either a C_{20} compound (Haworth *et al.* 1939; Hawkins & Hunter, 1944*b*) or a C_{25} ketone (Jones, 1951) or possibly both. In one or two of our experiments we obtained the former compound, but in most cases the only crystalline material which could be isolated after repeated chromatography was a C_{25} ketone of m.p. 79–80°. This showed $\lambda_{max.}$ 390 mμ. and $E^{1\%}_{1\,cm.}$ 1750–1800 (*cyclo*hexane) (Fig. 3). The position of the peak agrees closely with that of retinene$_2$ and that of the $C_{20}H_{26}O$ compound, but the absorption curve is narrower and $\epsilon_{max.}$ distinctly higher. The subsidiary maximum at 310 mμ. of retinene$_2$ is entirely absent.

Oppenauer oxidation of retinene$_1$

If the $C_{20}H_{26}O$ compound is derived from vitamin A by a two-stage dehydrogenation, retinene$_1$ is the likely intermediate. Accordingly, retinene$_1$ (1·0 g. cryst.) was refluxed continuously (48 hr.) with diethyl ketone (2 ml.), benzene (75 ml.) and aluminium *iso*propoxide (1·5 g.). The product was worked up as before. A compound of m.p. 77° was isolated, $\lambda_{max.}$ 387 mμ. $E^{1\%}_{1\,cm.}$ 1700 (*cyclo*hexane), 396 mμ. $E^{1\%}_{1\,cm.}$ 1610 (EtOH); 2:4-dinitrophenylhydrazone, m.p. 162°. The compound agreed with the $C_{20}H_{26}O$ material already described.

Oppenauer oxidation of vitamin A acetate

If it were possible in the reaction to dehydrogenate in the β-ionone ring, vitamin A_2 acetate or vitamin A_2 might have been formed and detected by the ultraviolet absorption maxima at 350 and 285 mμ. or by the $SbCl_3$ colour-test maximum at 693 mμ. In no case out of many trials on vitamin A acetate or natural esters was any evidence found for this change. The reaction products showed intense selective absorption with maxima at 415, 390, 370 and 350 mμ. reminiscent of anhydrovitamin A_1 or A_2, but not identical with either, since these substances do not show a 415 mμ. peak. The absorbing materials readily underwent polymerization to very pale amorphous solids which could not be crystallized.

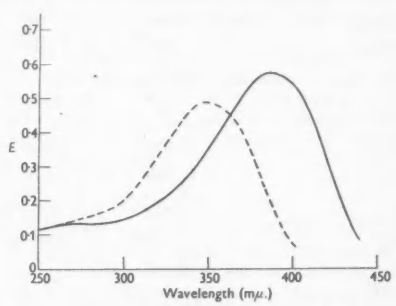


Fig. 4. Absorption spectrum of $C_{20}H_{26}O$ compound in light petroleum, ——; and reduction product ($LiAlH_4$) in light petroleum, – – –. $E^{1\%}_{1\,cm.}$ max. (at 386 mμ.) 1690.

Reduction of $C_{20}H_{26}O$ compound with lithium aluminium hydride

The compound was readily reduced to an alcohol with a flat peak maximum at 350–360 mμ. (ε value lower than that of the aldehyde, cf. Fig. 4). A band in this position is characteristic of vitamin A_2 but the secondary peak at 286 mμ. shown by the vitamin is absent (cf. Cama, Dalvi, Morton & Salah, 1952*a*). Although the reduction product is similar to vitamin A_2 it is clearly a distinct substance. This is borne out by the fact that its $SbCl_3$ colour-test maximum is at 720 mμ. (instead of 693 mμ.) and also by the failure to obtain from it anhydrovitamin A_2 ($\lambda_{max.}$ 393, 370, 350 mμ. $SbCl_3$ colour test 693 mμ.).

Reduction of $C_{20}H_{26}O$ *compound* in vivo

The crystalline compound was dissolved in diethyl ether, and small portions were added to arachis oil, and the ether removed by a current of N_2. Young newly weaned rats having low vitamin A reserves were given (orally, by syringe) arachis oil containing 20, 50 and 100 μg. of the C_{20} compound. The animals were killed by $CHCl_3$ 24 hr. later and the livers were ground with silver sand and anhydrous Na_2SO_4. The lipids were extracted with diethyl ether and the solvent removed after filtering. The residue was examined for ultraviolet absorption and by means of the $SbCl_3$ colour test.

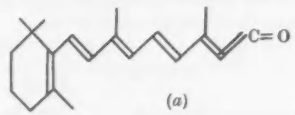
On no occasion was there any evidence of the formation of vitamin A_2. The C_{20} compound had undoubtedly been absorbed and probably reduced because the 720 mμ. colour-test peak was shown by the liver extracts and the 385 mμ. band in the ultraviolet absorption had disappeared.

DISCUSSION

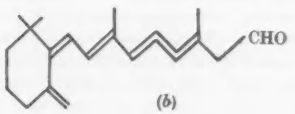
A compound $C_{20}H_{26}O$ (not crystallized) had been claimed by Haworth *et al.* (1939) to be an Oppenauer oxidation product of vitamin A on the basis of the properties of its oxime, m.p. 175–177°. Hawkins & Hunter (1944*b*) and Hunter (1950) clearly obtained the same oxime (m.p. 175–177°) by a similar route. So far as qualitative absorption spectra ($\lambda_{max.}$ 385 mμ.) and the $SbCl_3$ colour test ($\lambda_{max.}$ 730–735 mμ.) were concerned the $C_{20}H_{26}O$ compound might well have been retinene$_2$ (Morton *et al.* 1947), but Ponndorf reduction (Hawkins & Hunter, 1944*b*) did not give vitamin A_2 as it should have done on that basis. Instead, an alcohol ($\lambda_{max.}$ 350–370 mμ. $SbCl_3$ colour test 722 mμ.) was obtained and did not yield an anhydro compound analogous to anhydrovitamin A_2. As the structure attributed to the $C_{20}H_{26}O$ compound was that since attributed to retinene$_2$ (Morton *et al.* 1947) and established by Farrar *et al.* (1951) it was necessary to obtain the Oppenauer oxidation product pure. When crystallized (m.p. 77–78°) the material analysed correctly for $C_{20}H_{26}O$ and gave the oxime (m.p. 175–177°) obtained by previous workers (Table 2 and 3). The absorption peak ($C_{20}H_{26}O$) at 385 mμ. agreed with that of retinene$_2$, but the subsidiary maximum of the latter at 310 mμ. was lacking. The $SbCl_3$ colour test ($\lambda_{max.}$ 720 mμ.) did not agree with that of retinene$_2$ (735–705 mμ.) and on reduction *in vitro* and *in vivo* the distinction between the product and vitamin A_2 is definite.

This makes it very improbable that in the Oppenauer oxidation the second stage of dehydrogenation takes place in the β-ionone ring. The failure to obtain vitamin A_2 by Oppenauer oxidation of vitamin A acetate is confirmatory. Since, however, retinene$_1$ yields the $C_{20}H_{26}O$ compound, the first stage of dehydrogenation is the formation of vitamin A_1 aldehyde.

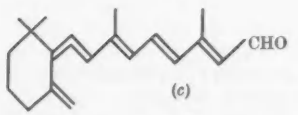
The structure of the $C_{20}H_{26}O$ compound is quite uncertain. Among the possibilities are:



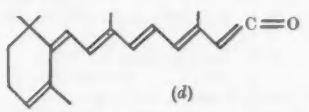
(*a*)



(*b*)



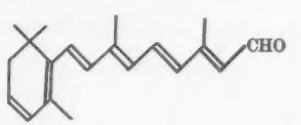
(*c*)



(*d*)

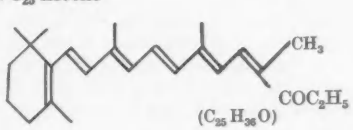
(*a*) being in our view the least likely. The unusual allene structures cannot, for lack of information on similar but simpler compounds, be justified on purely spectrophotometric evidence, although investigations of infrared or Raman spectra might prove useful (see Sheppard & Simpson, 1952).

The structure



belongs to retinene$_2$, but it is conceivable that a *cis*-isomer might be the $C_{20}H_{26}O$ aldehyde. The main objection to this is that neither prolonged heating nor catalysis with iodine showed any evidence of rearrangement to give retinene$_2$. Similarly, the reduction product ($LiAlH_4$) failed to isomerize to vitamin A_2.

The C_{25} ketone



($C_{25}H_{36}O$)

is presumably a direct condensation product of retinene$_1$ and diethyl ketone. It is, however, also possible that the $C_{20}H_{26}O$ compound may condense with diethyl ketone to give $C_{25}H_{34}O$. The former compound is quite easy to crystallize from light petroleum (m.p. 79–80°) and it shows a very high

$\epsilon_{max.}$. If, however, a similar compound is obtained from a reaction mixture containing the $C_{20}H_{26}O$ compound its properties suggest $C_{25}H_{34}O$, i.e. it derives from the $C_{20}H_{26}O$ compound.

SUMMARY

1. Oppenauer oxidation of vitamin A using diethyl ketone as hydrogen acceptor results in a complex mixture from which a compound $C_{20}H_{26}O$ (isomeric with retinene$_2$) can be isolated. It gives an oxime, m.p. 175–177°, isolated by previous workers.

2. The $C_{20}H_{26}O$ compound, is reducible *in vitro* and *in vivo*, but the product differs from vitamin A_2.

3. The Oppenauer oxidation seems to proceed via retinene$_1$, $C_{20}H_{28}O$, which can either be further dehydrogenated to $C_{20}H_{26}O$ or can condense with diethyl ketone, to a ketone $C_{25}H_{36}O$. Condensation of the $C_{20}H_{26}O$ compound with diethyl ketone to yield $C_{25}H_{34}O$ is not excluded.

4. Vitamin A acetate is not dehydrogenated directly to vitamin A_2.

We wish to thank the Medical Research Council for financial support.

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The Structure of Ribonucleic Acids

1. CYCLIC NUCLEOTIDES PRODUCED BY RIBONUCLEASE AND BY ALKALINE HYDROLYSIS

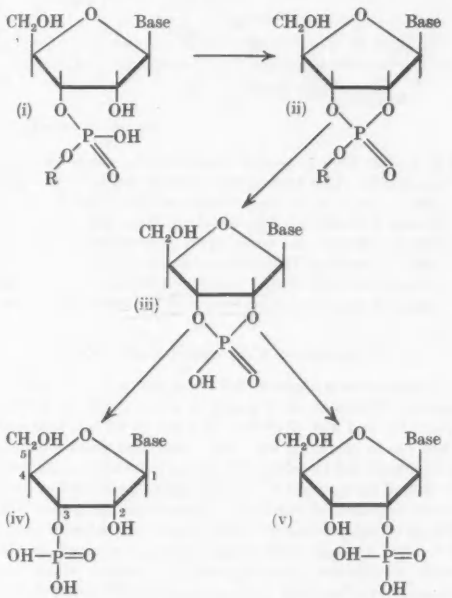
By R. MARKHAM and J. D. SMITH

Agricultural Research Council, Plant Virus Research Unit, Molteno Institute, University of Cambridge

(*Received* 20 *February* 1952)

One of the most striking chemical differences between the two main types of nucleic acid is that N-sodium hydroxide or potassium hydroxide at room temperature hydrolyses ribonucleic acids (RNA) rapidly and completely to nucleotides, but does not hydrolyse deoxyribonucleic acids in this way. The nucleotides produced on alkaline hydrolysis of RNA were thought for many years to be nucleoside 3′-phosphates (Levene & Harris, 1932), but recently Carter (1950), Cohn (1950) and also Loring & Luthy (1951) have demonstrated that all four ribonucleotides exist in the alkaline hydrolysates as pairs of isomers. These pairs, which have been called '*a*' and '*b*' nucleotides, differ in their chromatographic behaviour and are quite distinct from the nucleoside 5′-phosphates. They are stable to alkali, but may be interconverted by a short treatment with dilute acid (Brown, Haynes & Todd, 1950), and evidence has been given by Brown & Todd (1952*a*) that the '*a*' and '*b*' nucleotides are the nucleoside 2′- and 3′-phosphates, though not necessarily in that order, rather than α- and β-isomers (Doherty, 1950). The '*a*' and '*b*' nucleotides differ considerably in several properties such as solubility, and it is of interest to note that only the '*b*' forms of the pyrimidine nucleotides are liberated from RNA by ribonuclease, while at pH 8 a crude phosphatase from potato acts much more rapidly on the '*b*' form of adenylic acid than on the '*a*' form (Cohn, 1951; Kornberg & Pricer, 1950).

Working on the ribonuclease digestion of RNA (Markham & Smith, 1951*b*), we found that our digests contained a third type of nucleotide which belongs neither to the '*a*' or '*b*' series, nor to the group of nucleoside 5′-phosphates, but which could be converted to the former type by further treatment with ribonuclease. By a fortunate coincidence at about this time we had a discussion with Prof. A. R. Todd and several of his colleagues, and they were kind enough to describe their theory of the origin of the '*a*' and '*b*' nucleotides of Carter and Cohn to us (Brown & Todd, 1952*b*). This theory requires that the first step in the hydrolysis of a polynucleotide chain by alkali should be the formation of a tertiary phosphate ester (i → ii). This takes place by the esterification of the hydroxyl group on the sugar rings at carbon atom 2′ or 3′, whichever is free, by the primary phosphoric acid (—OH) group which is present in each internucleotide link.



Thus each phosphoric acid residue is in ester linkage with the hydroxyl groups at 2′ and 3′ of one nucleotide plus a hydroxyl group of the adjacent nucleotide in the chain. This structure is unstable and breaks down, leaving a series of 2′:3′-monohydrogen phosphate esters of nucleosides which, for convenience, we shall call 'cyclic' phosphates or 'cyclic' nucleotides (iii) in conformity with Brown & Todd. These substances are themselves unstable to alkali and break down, giving a mixture of the nucleoside 3′- and 2′-phosphates (iv and v). This theory, which is based upon the known properties of other phosphate esters, also explains the interconvertibility of the nucleoside 2′- and 3′-phosphates by acid, the alkali stability of deoxyribonucleic acids (which have no hydroxyl group at C—2′ and consequently cannot form a cyclic intermediate) and also suggests that the sugars which can form nucleic acids of the RNA type must have *cis* 2- and 3-hydroxyl groups.

Now it was quite evident from the data in our possession that cyclic nucleotides like (iii) would behave very much as did our nucleotides, and we were able to compare the synthetic and natural materials and demonstrate their identity. We have also managed to prepare all the four cyclic mononucleotides from RNA by careful alkaline hydrolysis and have thus been able to show that the scheme of Brown & Todd accurately represents the main steps in the alkaline hydrolysis of RNA.

EXPERIMENTAL

Materials

Commercial yeast RNA was reprecipitated and deproteinized (Smith & Markham, 1950). For the preparation of yeast RNA baker's yeast was treated with ethanol and ethanol-ether and dried. The dry yeast was then boiled at pH 8–8·4 in water for 20 min. An equal volume of ethanol was then added to the yeast suspension when cold and the precipitate was spun off. The supernatant liquid was acidified to pH 4·5 by addition of a small amount of acetic acid. The impure nucleic acid which precipitated was deproteinized (Sevag, Lackman & Smolens, 1938), precipitated with 50% (v/v) ethanol at pH 4·5 and dried.

Turnip yellow mosaic virus RNA was prepared as described previously (Markham & Smith, 1951*a*).

The 2′:3′-monohydrogen phosphates of cytidine, uridine and adenosine were given to us by Mr D. Magrath, who prepared them by a method to be published (Brown, Magrath & Todd, 1952).

Methods

Hydrolysis in presence of barium carbonate

RNA suspended in water was boiled under reflux in the presence of a small quantity of $BaCO_3$ for periods of up to 4 hr. (A useful time is 1 hr. at 100°.) Small amounts may be heated on a water bath in a stoppered tube. Barium carbonate, though very sparingly soluble in water, dissociates slightly in hot water to give a small amount of $Ba(OH)_2$, and this permits an easily controlled and mild alkaline hydrolysis. Solutions of NH_3 were also used at 50°, but this treatment tends to cause the production of nucleosides. In either case the yield of the cyclic phosphates is small.

Ribonuclease digestion

Solutions of crystalline ribonuclease were added to solutions of RNA at pH 7·5 and 20° in cellophan bags suspended in a large volume of water; the bags were agitated mechanically. The dialysate was collected at intervals and concentrated *in vacuo*. The dialysate contained large quantities of cyclic pyrimidine nucleotides, but only traces

of cyclic adenylic and guanylic acids, and then only from undegraded RNA. The reasons for this will be discussed in a later paper.

In preparing the cyclic nucleotides by this method, it is essential to keep the enzyme concentration low, so as to allow the nucleotides to escape further digestion by diffusing out of the bag. A suitable quantity of enzyme is 100 μg. in 10 ml. of a solution containing about 500 mg. of RNA.

Chromatographic solvents

Solvent 1. $(NH_4)_2SO_4$ sat. in water, 80 parts; 0·5 M-sodium acetate, 18 parts; *iso*propanol, 2 parts (v/v/v). *Solvent* 2. *iso*Propanol, 70 parts; water, 30 parts (v/v). *Solvent* 3. As solvent 2, with 0·35 ml. NH_3 solution (0·880 sp.gr.) added for each 1 l. of gas space in the tank and poured into the bottom of the tank. *Solvent* 4. *sec.*-Butanol sat. with water, 98%; glacial acetic acid 2%, (v/v).

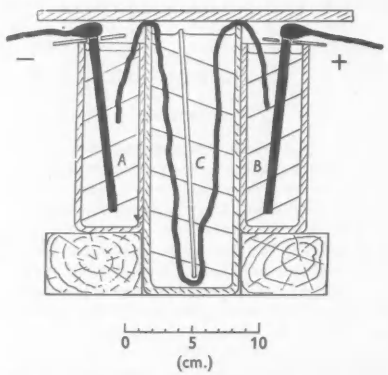


Fig. 1. The electrophoresis apparatus. Three standard museum jars (Baird and Tatlock, Ltd.: *A* and *B*, 6 × 4 × 2 in., *C*, 8·25 × 4·63 × 2·5 in.) were set up as shown, *A* and *B* containing buffer solution and *C* containing CCl_4 to cool the paper. The carbon electrodes were insulated at the top with tape after attaching the high-tension leads. The plate-glass lid covers the electrodes for safety. The spacer in jar *C* is celluloid, as are the two plates at the top of the electrodes.

Electrophoretic separation

The apparatus, which has been described briefly (Markham & Smith, 1951*c*), is shown in Fig. 1. The paper, a strip of Whatman no. 3 paper, 56 × 8 cm., is soaked in 0·05 M-ammonium acetate or formate buffer, and blotted to remove surplus moisture. The mixture to be run is put on a suitable place, usually about 12 cm. from the negative end, in a line at right angles to the long axis of the paper. About 0·1–0·2 ml. of solution is used. The paper is then immersed in CCl_4 in vessel *C* and the ends are allowed to dip into vessels *A* and *B* which contain 0·05 M-buffer. CCl_4 is essential as it prevents heating and evaporation and so the rates of movement of the bands are uniform and reproducible. The CCl_4 also prevents liquid siphoning over into the central part and waterlogging the paper. It is also transparent to the ultraviolet light used to detect the bands, and being non-polar has no tendency to dissolve out the nucleotides.

A potential of 1000 V. is applied to the ends of the paper, and a suitable power supply is shown in Fig. 2. The current used is 8–10 ma. with the buffers mentioned and an average run takes 2 hr. If phosphate buffers are used the potential should be dropped to 700 V. to prevent overheating. It is perhaps unnecessary to mention that the power supply is dangerous.

We use ammonium acetate and formate buffers as the components travel as spots on chromatograms. It is therefore unnecessary to desalt material removed from the electrophoresis strips before chromatography.

Location of the nucleotides. The nucleotides are located on the chromatogram and electrophoresis strips by ultraviolet photography (Markham & Smith, 1949, 1951*a*). When Whatman no. 3 paper is used the photographic exposure is 3–5 min.

Quantitative analysis. The purine and pyrimidine content of preparations was determined by a chromatographic method (Markham & Smith, 1951*a*). The phosphorus analyses were made by the method of Allen (1940).

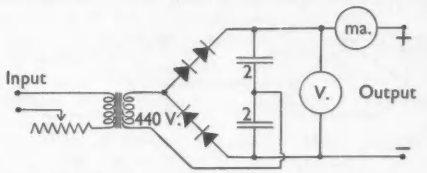


Fig. 2. The 1000 V. power supply for the electrophoresis apparatus. The transformer output must be at least 440 V. r.m.s., at 60 ma. or more (a 250–0–250 V. transformer is suitable). The metal rectifiers are in pairs of 350 V., 60 ma., in series. The condensers are 2 μF., 1000 V. working. The series resistance in the transformer primary is 1000–5000 Ω rated at 200 ma. and may be replaced with advantage by a variable autotransformer.

Preparation of the cyclic nucleotides

A ribonuclease digest of RNA was put on to the top of a piece of Whatman no. 3 paper in a line 25 cm. long. The quantity used was 10–20 mg. of digest in 0·5 ml. This was then run in solvent 3 for such a time that yeast adenylic acid would have run about 15 cm. (the solvent being allowed to run off the notched end of the paper). It was found that there were several bands of substances on the paper. The lowest of these (band 6), which has an R_F value of about 1·7 times that of yeast adenylic acid, contained all the cyclic nucleotides excepting that of guanine, which was found above (band 5*a*) with an R_F about 1·35 times that of yeast adenylic acid. Cyclic adenylic acid and guanylic acids will only be found in digests of good RNA and then only in minute amounts. The other bands contained substances which will be described in the following paper.

Cyclic pyrimidine nucleotides. These may be obtained in quantity from band 6 and may then be resolved from each other by: (*a*) paper chromatography in solvent 2, in which the cyclic uridylic acid has a higher R_F value than the cyclic cytidylic acid or (*b*) chromatography in solvent 4 which gives smaller R_F values but the same separation. Solvent 4 can, in fact, resolve the cyclic pyrimidine nucleotides from digests of commercial RNA directly, the other components having negligible R_F values. The cyclic pyrimidine nucleo-

tides can also be separated by electrophoresis for 2 hr. at 1000 V. and pH 3·5 when the uridylic acid will move about 16 cm. and the cytidylic acid about 6·5 cm. towards the positive electrode.

Cyclic purine nucleotides. Cyclic purine nucleotides comprise only about 2% of the total material in a yeast RNA digest and are not easy to demonstrate in such digests. Commercial yeast RNA which we have used yielded no cyclic purine nucleotide on ribonuclease digestion but after $BaCO_3$ hydrolysis these could be isolated in small yield. The $BaCO_3$ hydrolysate is best further digested with ribonuclease to remove the cyclic pyrimidine nucleotides and then separated on paper in solvent 1. Band 5*a* is almost pure cyclic guanylic acid and band 6 cyclic adenylic acid. These may be further purified by electrophoresis, when the contaminating nucleosides are left behind. The adenylic acid will move about 8 cm. and the guanylic acid about 14 cm. in 2 hr. at 1000 V. and pH 3·5.

RESULTS

Cyclic pyrimidine nucleotides

Cyclic uridylic acid has been analysed for phosphorus and uracil, which are present in the molar ratio 0·95:1 (theory 1:1). The absorption spectrum

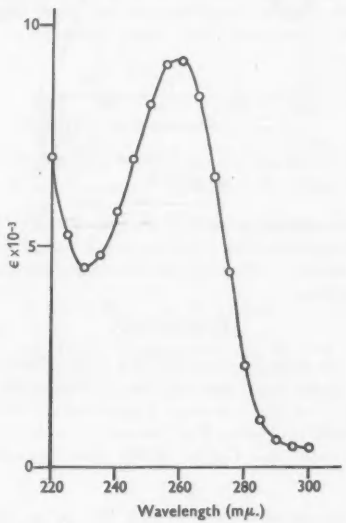


Fig. 3. The absorption spectrum of cyclic uridylic acid at pH 7·3.

is shown in Fig. 3. Cyclic cytidylic acid has a molar ratio of cytosine/P of 0·93:1 (theory 1:1). The absorption spectrum is shown in Fig. 4. The chromatographic behaviour is identical with that of the synthetic material (which was contaminated with the non-cyclic form of the nucleotides) in all solvent systems used (solvents 1–4 and several others).

Perhaps the most striking proof of the identity of the natural and synthetic material is the fact that both are hydrolysed slowly to the free nucleotides by crystalline ribonuclease. In this hydrolysis only the '*b*' isomer is formed (Brown, Dekker & Todd, 1952).

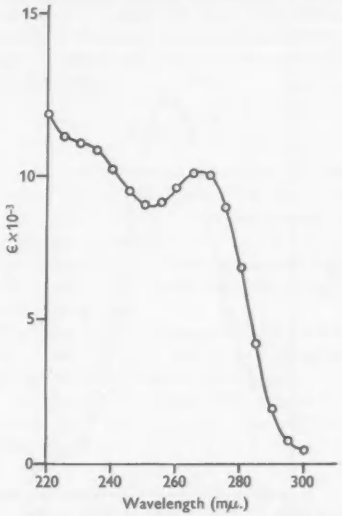


Fig. 4. The absorption spectrum of cyclic cytidylic acid at pH 7·3.

Fig. 5. The electrophoretic separation of the cyclic cytidylic acid and ordinary cytidylic acid obtained from the former by ribonuclease digestion. Strip run for 1 hr. in phosphate buffer, pH 7·4, at 20 V./cm. The cyclic form having one less OH group moves more slowly. The white line shows where the spots were put on.

After hydrolysis it is possible to demonstrate the presence of an additional acidic group dissociating at a pH between 3·5 and 7, by electrophoresis of the material at pH 7·4 (Fig. 5). This group is, of course, a secondary phosphoric acid —OH, which is not to be found in the cyclic forms (see formulae iii–v).

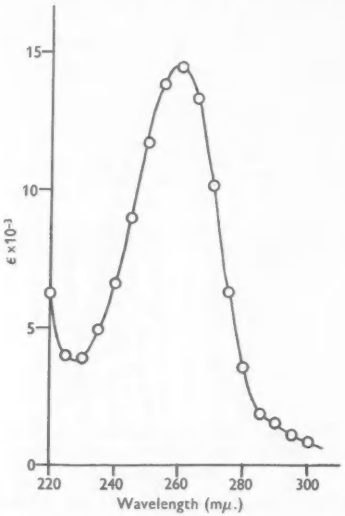


Fig. 6. The absorption spectrum of cyclic adenylic acid at pH 7·3.

At pH 3·5 the two forms have the same mobility. The properties of the cyclic and non-cyclic pyrimidine nucleotides are summarized in Table 1.

Cyclic purine nucleotides

The molar ratio phosphorus/adenine in the cyclic adenylic acid has been found to be 1·00:1 (theory 1:1). The absorption spectrum is shown in Fig. 6.

The molar ratio phosphorus/guanine in the cyclic guanylic acid has been found to be 0·93:1 (theory 1:1). The absorption spectrum is given in Fig. 7.

The cyclic adenylic acid has been compared with synthetic material (which was only about 80% pure), but the guanylic acid derivative has only been identified by analogy, i.e. general electrophoretic and chromatographic properties.

The general properties of the cyclic purine nucleotides are similar to those of the corresponding pyrimidine nucleotides with the important exception that they are *completely resistant to ribonuclease*. On hydrolysis with cold N-hydrochloric acid or N-sodium hydroxide, they give rise to

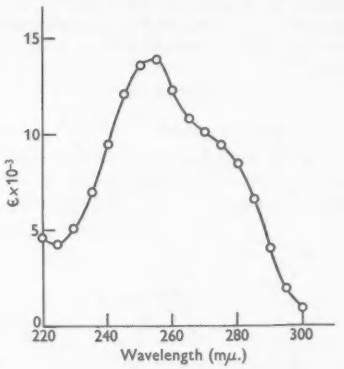


Fig. 7. The absorption spectrum of cyclic guanylic acid at pH 7·3.

mixtures of the '*a*' and '*b*' nucleotides, which may be demonstrated by running in solvent 1. Table 1 also includes a summary of the properties of these nucleotides.

DISCUSSION

From the data presented in this paper there can be little doubt that the scheme of Brown & Todd (1952*b*) for the alkaline hydrolysis of RNA is essentially correct. The isomeric nucleotides of Cohn (1950) and Carter (1950) must therefore be

Table 1. *The properties of the cyclic nucleotides contrasted with those of the analogous nucleoside 2′- and 3′-phosphates*

Cyclic nucleotides	Nucleoside 2′- and 3′-phosphates
Greater R_F value in solvent 3	Smaller R_F value in solvent 3
Smaller R_F value in solvent 1	Larger R_F value in solvent 1
Same electrophoretic mobility at pH 3·5	Same electrophoretic mobility at pH 3·5
Smaller relative electrophoretic mobility at pH 7·4	Larger relative electrophoretic mobility at pH 7·4
Pyrimidine derivatives hydrolysed slowly by ribonuclease	Unaffected by ribonuclease
Hydrolysed exceedingly slowly by prostate phosphomonoesterase	Dephosphorylated rapidly by phosphomonoesterase
Hydrolysed by cold N-NaOH to give a mixture of the nucleoside 2′- and 3′-phosphates	Unaffected by cold N-NaOH

regarded as artifacts of hydrolysis. The question still remains as to which of the two types of nucleotide, the 3′ or the 2′ nucleotide, is the natural form. By analogy with deoxyribonucleic acid, the 3′ type is the natural choice, but a definite solution of this problem will probably demand some new approach, and, of course, it is by no means certain that all the nucleotides will have the same configuration, though this would seem likely.

From the evidence which we have presented here, it might be thought that our 'cyclic' nucleotides might be dinucleotides or pyrophosphates. As we shall describe the isolation and properties of both cyclic and non-cyclic dinucleotides in the next paper, it may suffice here to mention that dinucleotides have smaller R_p values in all solvent systems, a higher electrophoretic mobility at all pH values, and, of course, are only half dephosphorylated by phosphomonoesterase.

The most striking point emerging from these experiments is the evident analogy between the action of ribonuclease and that of alkali on RNA, and the discrimination of the former between purine and pyrimidine nucleotides. This is in keeping with the specificity of ribonuclease, which we shall describe in detail later. At this point it may be pertinent to remark that, as we believe that the mechanism of action of ribonuclease on the cyclic pyrimidine nucleotides is the same as that of the enzyme on intact RNA, the cyclic nucleotides, which may be synthesized or isolated in adequate amounts, are obvious substrates for kinetic and other studies on ribonuclease, particularly as the reaction is of a simple type while, as the rate of reaction is relatively slow, it may be followed without difficulty.

SUMMARY

1. Cyclic nucleotides (2′:3′-monohydrogen phosphate esters of nucleosides) have been obtained from ribonucleic acids by ribonuclease action and by careful alkaline hydrolysis.

2. The properties of these substances have been described and they have been compared with synthetic specimens.

3. The cyclic pyrimidine nucleotides are digested by ribonuclease to give ordinary nucleotides while the analogous purine derivatives are not.

4. A method and apparatus for separating these substances by paper strip electrophoresis is described.

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The Structure of Ribonucleic Acids

2. THE SMALLER PRODUCTS OF RIBONUCLEASE DIGESTION

By R. MARKHAM and J. D. SMITH
Agricultural Research Council, Plant Virus Research Unit, Molteno Institute, University of Cambridge

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For many years it was thought that nucleic acids were tetranucleotides. This was largely due to observations such as those of Levene (1909) who showed that the ribonucleic acid (RNA) of yeast had approximately equivalent amounts of the two purines adenine and guanine and the pyrimidines cytosine and uracil. Analytical methods of that period were incapable of distinguishing small differences in the proportions of the bases, while the absence of techniques for isolating RNA in a native condition and for estimating the order of size of the molecules, was responsible for the failure of workers to realize that RNA was a much more complicated substance than could be represented by a chain of four nucleotides. Much effort was expended in testing this theory, for example by titration studies, made for the most part on degraded material and probably involving further degradation during the process. However, such experiments and those involving the action of enzymes led to the belief that RNA was a larger molecule having a 'statistical' rather than a 'structural' tetranucleotide composition (Gulland, Barker & Jordan, 1945). This hypothesis itself did not fit the observed facts, and the advent of chromatographic techniques has led to its complete abandonment. Reliable analyses for several ribonucleic acids are now available, and these fully confirm the earlier suspicions about the invalidity of the tetranucleotide hypothesis (Markham & Smith, 1950, 1951*a*; Smith & Markham, 1950).

Although much is known about the gross analytical chemistry of RNA much less is known about the nature of the internucleotide links. Cold alkaline hydrolysis leads to the formation of the '*a*' and '*b*' nucleotides (Carter & Cohn, 1950) which have been shown by Brown & Todd (1952) to be the nucleoside 2′- and 3′-phosphates (though not necessarily in that order). Cohn & Volkin (1951) have demonstrated the presence of nucleoside 5′-phosphates in digests after treatment of RNA with ribonuclease plus phosphodiesterase, thus showing that the 5′-hydroxyl group also participates in the internucleotide link. The quantity of 5′-phosphates isolated by these authors suggests that a large number, if not all, of the internucleotide linkages are through the sugar residues.

In the following paper we shall describe the smaller fragments produced by ribonuclease digestion—the mono-, di- and tri-nucleotides, their separation and their structure—and we hope to demonstrate that one kind of phosphate diester linkage is common to all.

Nomenclature

As we shall be dealing in this paper with a large variety of complex molecules, we have adopted a shorthand method of notation. As it is at present impossible to differentiate with certainty between the nucleoside 2′- and 3′-phosphates, we shall assume for convenience that any link involving one of these positions, is a 3′-hydroxyl derivative. Adenylic, guanylic, cytidylic and uridylic acids are represented by A, G, C and U respectively, while their (cyclic) monohydrogen phosphate derivatives (Markham & Smith, 1952) are represented by A!, G!, C! and U! respectively. In a dinucleotide the order in which the nucleotides are written is such that the second nucleotide has a free 3′-phosphate, or the equivalent, and is linked by its 5′-hydroxyl group to the preceding nucleotide. Thus GU! is the 2′:3′-cyclic uridylic acid 5′-ester of guanosine 3′-phosphate (the 5′-carbon being the only free position for the ester link in the cyclic uridylic acid), while GU is the similar dinucleotide containing uridine 3′-phosphate. UG, on the other hand, would represent the guanosine 3′-phosphate, 5′-ester of uridine 3′-phosphate, which, incidentally, is not found in ribonuclease digests.

MATERIALS AND METHODS

The nucleic acids used were prepared as in the preceding paper.

An impure but otherwise excellent turnip-yellow-mosaic-virus nucleic acid was also prepared by denaturing the virus in neutral solution with an equal volume of neutral ethanol, spinning off the protein and precipitating the nucleic acid by increasing the ethanol concentration to 75% (v/v). The preparation was then dried at room temperature. In this way one can be sure that no damage has been done to the acid- and alkali-labile groups in this nucleic acid.

Ribonuclease. Ribonuclease was obtained in a crystalline form from two sources and both preparations had very similar properties.

Phosphomonoesterase. Phosphomonoesterase was prepared from hypertrophic human prostate glands by grinding them with sand in 0·9% NaCl solution and allowing the suspension to autolyse under toluene for 18 hr. at 37°. The liquid was then freed from solids by centrifuging and the fraction of material precipitating between 0·5 and 0·75 saturation $(NH_4)_2SO_4$ was collected. This was further purified by salt precipitation followed by dialysis. The centrifuged solution was then stored in 0·1 M-acetate buffer, pH 5, with a few drops of $CHCl_3$ added to prevent bacterial growth. This enzyme dephosphorylated nucleotides and glycerophosphate rapidly, but had no detectable ribonuclease or diesterase activity.

Chromatographic solvent systems. For chromatographing we used four main solvent systems. (1) The *iso*propanol-ammonia system of the preceding paper (Markham & Smith, 1952), (2) sat. $(NH_4)_2SO_4$, 80 parts; *iso*propanol, 2 parts; 0·5 M-sodium acetate, 18 parts (v/v/v). (Markham & Smith, 1951*a*), (3) *n*-butanol saturated with water, 90 parts; formic acid, 10 parts (v/v) (Markham & Smith, 1949), and (4) *iso*propanol, 170 ml.; conc. HCl, 44 ml.; water to 250 ml. (Wyatt, 1951). Solvent 3 was used for detecting nucleosides, while solvent 4 was used for estimating the composition of the various fractions after hydrolysis in N-HCl for 1 hr. at 100° to give the purines and the pyrimidine nucleotides.

Ribonuclease hydrolysis. Hydrolysis was carried out as described in the previous paper. It will be appreciated that, as many of the substances isolated are themselves either rapidly or slowly digested by ribonuclease, one must be prepared to accept very small yields. On the other hand, if only the stable di- and tri-nucleotides are to be isolated, then the enzymic digestion may be allowed to go to completion. The dialysates from the digest were concentrated *in vacuo* and then chromatographed.

Analysis of fractions. The polynucleotides and mononucleotides when hydrolysed in N-HCl at 100° for 1 hr. give free purines and pyrimidine nucleotides which could then be identified and analysed quantitatively by chromatographing in solvent 4 (Markham & Smith, 1951*a*).

The internucleotide links were broken by treating the substances with N-NaOH or N-HCl overnight or by boiling in the presence of $BaCO_3$. The components could then be identified by running in solvents 2 and 4.

RESULTS

The initial chromatography

The digest (10–20 mg.) was put on to Whatman no. 3 paper in about 0·5–1 ml. of solution. This solution was applied in a line across the top of the paper about 6 cm. from the top edge, and some 25 cm. long. The paper was cut into notches at its lower end, and was then developed in solvent 1 until the band of the cyclic pyrimidine nucleotides nearly reached the bottom. (This may be observed quite easily by examining the chromatograms in ultraviolet light through a blue filter held before the eye, when the bands will appear dark (see Markham & Smith, 1951*a*, p. 402).) After drying off the solvent the chromatogram was printed on to photographic paper as previously described (Markham & Smith, 1949) but for a longer period (3–5 min.). A typical result is shown in Fig. 1. To obtain this result one may have to make minor adjustments to the solvent system, the ammonia concentration being the most important factor. The bands once located were cut out and eluted by allowing water to run along them. About 1 ml. of the extract was collected from each strip and this was then evaporated to dryness in a current of air at a température not exceeding 50°. This procedure fractionated the mixture into six main fractions numbered 1–6 in order of R_F values (Fig. 1). These fractions were not very sharp, but it must be remembered that there may be more than twenty individual substances on the paper.

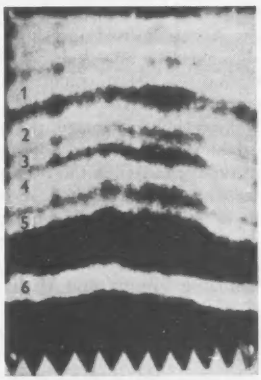


Fig. 1. The initial chromatogram. The bands are numbered as in the text. Band 4 is in the position to which yeast adenylic acid runs. Band 5*a* is not present in this particular chromatogram.

This chromatographic separation depends upon several factors. All guanine compounds move more slowly than do the similar compounds of the other bases, all cyclic derivatives have higher R_F values than the similar 2′- and 3′-phosphates, and mononucleotides move faster than dinucleotides, which in turn move faster than trinucleotides. This order is tabulated in Table 1.

Paper electrophoresis

The apparatus used has been described in detail in the first paper of this series, but it is necessary here to give a short account of certain theoretical points connected with its use.

The four nucleotides have each several ionizing groups, namely the primary and secondary OH groups of the phosphoric acid residues, the NH_2 groups of A, G and C, and the enolic OH groups of G, C and U. The last need not concern us here, but the first three are very important. The pK′ values (Levene & Simms, 1925) of the primary

phosphoric OH groups range from 0·7 to 1·02, so that for all practical purposes they can be regarded as fully dissociated at any pH value at which the internucleotide link is stable. The pK′ values for the NH_2 groups, on the other hand, are 3·7 for A, 2·3 for G and 4·24 for C, while U of course has none. Consequently, it is possible to select a pH value to take advantage of the net charges on the nucleotides to effect their separation. A useful pH value for this purpose is 3·5, though this is not suitable for all separations.

At pH values above 7, the secondary phosphoric OH groups are fully dissociated, so that compounds lacking these groups, i.e. polynucleotides containing cyclic nucleotides as end groups, or cyclic nucleotides themselves, can be distinguished. It is also possible to eliminate the effects of the NH_2 groups simply by raising the pH to a suitable value, i.e. above pH 5 for C and above pH 3·5 for G.

The electrophoretic behaviour of polynucleotides is a subject which we have already discussed briefly from a theoretical standpoint (Markham & Smith, 1951*b*). The rate at which a molecule moves through a fluid when it is exposed to a voltage gradient depends upon two main factors: (*a*) the net charge, which is the algebraic sum of the products of the number of ionizing groups and their percentage dissociation, and (*b*) the resistance to motion through the fluid. Nucleotides are rather asymmetrical molecules, and from the diffusion data of Gordon & Reichard (1951) it would appear that they have an axial ratio of about 5:1. Consequently a dinucleotide will probably have an axial ratio of between 2·5:1 and 10:1. Using these limits and assuming that the resistance to motion is the same as in translational diffusion, it is possible to set limits for the electrophoretic mobility of a nucleotide dimer as opposed to a monomer. The dimer has, of course, twice the charge per molecule, and rather less than twice the resistance to motion, so that the net effect is for any dimer to have a higher mobility than its monomer. We assess these limits as between 1·32 and 1·85 times that of the monomer, and for our preliminary work we took 1·54 as the most likely value. Since then we have been able to measure the actual values for the mono-, di- and trinucleotides of uracil and get a ratio of 1:1·27:1·37 for these substances; but these values contain an error in that the endosmotic flow is in the direction of motion, and consequently the absolute values of these ratios will actually be rather higher. A rough assessment of the amount of endosmotic flow suggests that about 25% of the movement of uridylic acid is due to this cause at pH 3·5, and the ratios of the actual mobilities thus become about 1:1·4:1·5. It will be seen that this series converges, so that a separation of tetra and higher nucleotides is unlikely.

In actual use, the electrophoretic separation has been simplified in that a preliminary fractionation has been achieved by chromatography, so that the system is not very complex. It is very useful, however, for the identification of various substances, to make use of the possibility of calculating relative mobilities, always bearing in mind that the method is to some extent empirical. As an example, take the relative net negative charges of the mononucleotides at pH 3·5. These are A, 0·46, G, 0·95, C, 0·16, and U, 1·00. From these one can calculate the mobilities relative to U of some di- and tri-nucleotides. Thus the mobility towards the positive electrode of

$$\text{AG} = (0{\cdot}46 + 0{\cdot}95)/1{\cdot}43 = 0{\cdot}99,$$
$$\text{of AC} = (0{\cdot}46 + 0{\cdot}16)/1{\cdot}43 = 0{\cdot}43,$$
$$\text{of AGC} = (0{\cdot}46 + 0{\cdot}95 + 0{\cdot}16)/2 = 0{\cdot}79,$$
$$\text{and of AAU} = (0{\cdot}46 + 0{\cdot}46 + 1{\cdot}0)/2 = 0{\cdot}96,$$

times that of uridylic acid at pH 3·5. The factors 1·43 and 2 represent the increased friction to motion of the dimers and trimers. (It is assumed that to a close approximation all the mononucleotides are about the same size.) With these figures it is possible to calculate the relative mobility of any mono-, di- and tri-nucleotide at this pH. For any other pH value, it is only necessary to calculate the charges for the four nucleotides from the appropriate pK′ values and proceed as above. It will be noticed that at this pH small inaccuracies in making up the buffer may make quite large differences to the mobility. In practice this is relatively unimportant.

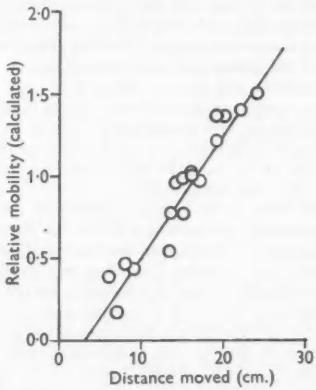


Fig. 2. Graph of mobility observed (in cm./2 hr./20 V./cm.) against relative mobility calculated from the charge and friction, for 19 mono- and poly-nucleotides (two of the points are on top of others).

Fig. 2 shows the extent to which one may rely on measurement of mobility for the identification of unknown nucleotides. This was compiled from a large number of different runs made for preparative

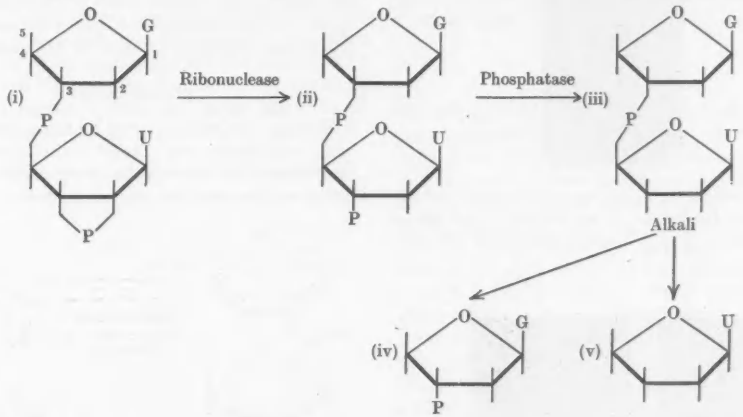
purposes, and no particular efforts were made to control the voltage gradient to better than ± 10 %.

For most purposes a run of 2 hr. with a gradient of 20 V./cm. at pH 3·5 is adequate. This gradient is given to a close approximation if a strip of filter paper 57 cm. (22·5 in.) long is used in the apparatus, the ends being allowed to dip into the electrode vessels about 3 cm. below the surface of the buffer and a total potential of 1000 V. is applied to the electrodes.

Cyclic dinucleotides

The isolation of the cyclic dinucleotides and their characterization is best illustrated by one example and for this we will take GU!. This substance was found in band 3 (Fig. 1) which, when dissolved in buffer and subjected to electrophoresis at pH 3·5 for 2 hr. at 20 V./cm. gave two bands moving about 20 and 15 cm. respectively. The former is GU!, the latter GC!.

band 3 was in fact GU! (i) which on ribonuclease treatment went to GU (ii). This was confirmed by running GU! before and after ribonuclease treatment in the electrophoresis apparatus at pH 7·4 (Fig. 3), when it was seen that digestion liberates an additional acidic group with a pK′ of between pH 4·5 and 6, i.e. a secondary phosphoric OH. This finding does not exclude the possibility of the secondary phosphoric OH being attached to the G residue, i.e. the substance being UG!, but this is unlikely as G! itself is not attacked by ribonuclease. The confirmation of the structure came from two different experiments. GU prepared from GU! by ribonuclease treatment gave on hydrolysis with barium carbonate a quantity of G!. GU on treatment with phosphomonoesterase gave rise to a new substance (iii) which had quite different properties (R_F value higher in solvent 1 and electrophoretic mobility much smaller) and which on hydrolysis in N-sodium hydroxide at room temperature gave rise



When analysed GU! was found to contain approximately equivalent amounts of guanine and uridylic acid (found: 0·9 mole/1·0 mole), and after alkaline hydrolysis was shown, by chromatography in solvent 2, to give guanylic acids '*a*' and '*b*' and a pyrimidine nucleotide. When treated further with ribonuclease, the dinucleotide gave a substance still containing equivalent amounts of guanine and uridylic acid (found: 0·9 mole/1·0 mole) but running in solvent 1 in the position of band 1. The R_F values in solvent 2 were reversed in order. This behaviour is similar to that of the cyclic pyrimidine nucleotides which, as we have shown in the previous paper, are digested by ribonuclease to liberate nucleoside 3′-phosphates. The rate of this digestion is much smaller than that of the internucleotide linkages and consequently it is possible to isolate these intermediates. So it was probable that the substance in

to equivalent quantities of guanylic acid (iv) and uridine (v). (iii) belongs to a new class of compounds, the dinucleoside monophosphates. There can therefore be no doubt that (i) represents the formula of GU! and (ii) that of GU.

Other dinucleotides

GC!, as we have already mentioned, was also found in band 3 (Fig. 1). On ribonuclease treatment it gave GC, which is to be found in band 1, and phosphatase subsequently converted it to the cytidine ester of guanylic acid. The analysis of GC gave G/C = 0·99 mole/1 mole. The separation of GC will be considered under trinucleotides.

AC and AU were found in chromatographic band 2 and moved about 8 and 16 cm. respectively in 2 hr. at 20 V./cm., on electrophoresis at pH 3·5 (Fig. 4). Analyses gave A/C = 1·03 moles/1 mole

Fig. 3. Electrophoresis at pH 7·4 of GU! before and after ribonuclease treatment. It will be noted that the GU formed has an extra (secondary phosphoric OH) acidic group.

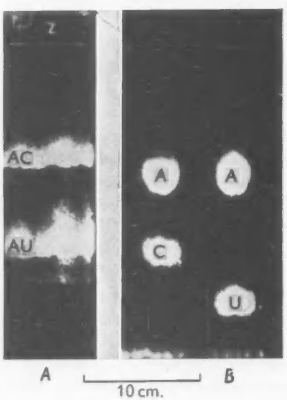


Fig. 4. Isolation and analysis of AC and AU. (*A*) The electrophoresis strip of band 2 (Fig. 1) showing AC and AU and a trace of GU from band 1; (*B*) the dinucleotides hydrolysed in N-HCl at 100° for 1 hr. and chromatographed in solvent 4, showing the presence of adenine in both and cytidylic acid in AC and uridylic acid in AU.

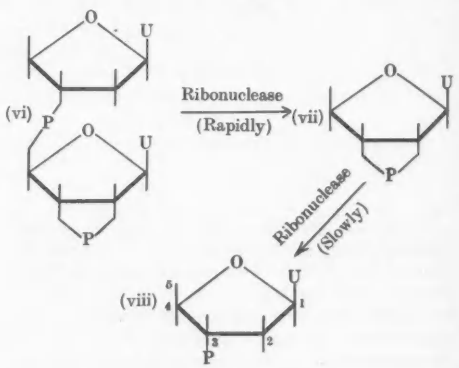
and A/U = 0·95 mole/1 mole. The analogous AC! and AU! were in chromatographic band 5.

The molar ratio of A/U! was found to be 0·97/1 and that of A/C! was 1·1/1. AC! and AU! were converted to AC and AU by ribonuclease, and the latter had only their pyrimidine nucleotide residues dephosphorylated by phosphatase, so that their structures are similar to that of GU. These dinucleoside phosphates have been isolated, hydrolysed in N-sodium hydroxide and degraded to purine nucleotides '*a*' and '*b*' and the appropriate pyrimidine nucleoside. The ratio of total purine nucleotide to pyrimidine nucleoside was found to be approximately 1/1 in all three cases.

AG (or GA) was found in band 1 (Fig. 1) in digests of commercial yeast nucleic acid. Its absence from carefully prepared specimens of nucleic acid suggests that it was an artifact of partial degradation of the nucleic acid, probably by alkali, which leaves this dinucleotide at one end of a short-chain fragment. The analysis gave a ratio of A/G = 1·1 moles/1 mole. It has not been subjected to phosphatase treatment and it may well be a mixture of AG and GA.

Diuridylic acid

Perhaps the most interesting dinucleotide is UU! This substance was found in chromatographic band 5 with the other cyclic non-guanine-containing dinucleotides, and its electrophoretic mobility at



pH 3·5 was approximately 1·4 times that of U and U!. UU itself was not found in the digests, as the internucleotide link breaks before the cyclic phosphate link. Consequently UU! (vi) was converted entirely to U! (vii) by ribonuclease before any U (viii) was liberated, and UU! was converted by both acid and alkali to U only, thus proving conclusively that the alkali-labile link between two pyrimidine nucleotides was the same as that between purine nucleotides and pyrimidine nucleotides (as in AU, etc.).

In addition to UU!, CC! was also found, particularly in digests of the turnip-yellow-mosaic-virus nucleic acid (which has a very high proportion of cytidylic acid), and we have also detected CU! (or UC!). These substances were found in chromatographic band 5, but little work has been done on them, as they are much more difficult to prepare than UU! and are only present in very small amounts.

Trinucleotides

The trinucleotides were found in chromatographic bands 1 and 2 with the exception of UUU!, which was in band 5.

UUU! has a mobility of 24 cm./2 hr. at pH 3·5 with a voltage gradient of 20 V./cm., and as may be expected was found in very low yield. Its identification is based upon the fact that it contains only uridylic acid, and must therefore be a polyuridylic acid. If it is assumed that each internucleotide link in such a substance is equally susceptible to ribonuclease, it will be seen that the probability of the higher polymers existing in any quantity decreases rapidly with chain length, so that the absence of a band between UU! and what we call UUU! makes it exceedingly unlikely that the latter has a more complex structure.

Table 1. *The chromatographic and electrophoretic properties of mono-, di- and tri-nucleotides*

Nucleotide	Chromatogram band no. (Fig. 1)	Mobility in cm./2 hr. at pH 3·5 and 20 V./cm.
A	4	8
G	2	14
C	4	6·5
U	4	16
A!	6	8
G!	(5*a*)	14
C!	6	7
U!	6	16
AC!	5	9
AU!	5	16
UU!	5	22
AC	2	8
AU	2	16
AG	1	15
GC!	3	15
GU!	3	20
GC	1	13·5
GU	1	19·5
UUU!	5	24
ACC!	2	6
AAC	1	13·5
AAU	1	17
AGU	1	19

Chromatographic band 1 subjected to electrophoresis at pH 3·5 for 2 hr. at 20 V./cm. gave rise to electrophoretic bands at (*a*) 13·5, (*b*) 17 and (*c*) 19 cm. (as well as one at 21 cm. which is GU). Band *a* was a mixture of GC and AAC. This could be resolved into its components by eluting and rerunning at pH 2·5 at 16 V./cm. for 3 hr., when GC moved 4·8 cm. and AAC moved 6·4 cm., or by running again at pH 5·0 for 3 hr. at 20 V./cm., when AAC ran just ahead (28 cm. against 24·5 cm.). The analysis of AAC gave a ratio of A/C = 1·92 moles/1 mole (theory 2/1). Band *b* was AAU (molar ratio A/U = 1·81/1). Band *c* was AGU (molar ratio A/G/U = 0·95/1/1·15). This trinucleotide could, of course, be GAU or a mixture of both AGU and GAU, but for reasons which we will discuss later it is probable that the uridylic acid residue is at one end of the molecule linked by its 5′-hydroxyl group.

Chromatographic band 2 subjected to electrophoresis at pH 3·5 for 2 hr. at 20 V./cm. sometimes gave a band moving 6 cm. This was ACC! (A/C = 1 mole/1·97 moles).

The positions and properties of these and some other substances are given in Table 1 which should prove an adequate guide to the isolation and identification of the various nucleotides.

DISCUSSION

The results presented in this paper and the previous one are all consistent with the hypothesis that those linkages in RNA which are broken by ribonuclease to give mono-, di- and tri-nucleotides, as well as the linkages in the latter which are broken by alkali, are all of the same type, i.e. a series of secondary phosphate ester links joining adjacent nucleosides through the hydroxyl groups at carbon atoms 3′ (or possibly 2′) and 5′. The link at C-5′ is evidently that which is broken by alkaline hydrolysis.

The end products of ribonuclease digestion have, with the exception of the mononucleotides and of AG, which is only obtained from chemically degraded RNA and is evidently an artifact, one and only one pyrimidine nucleoside residue per molecule, which is in the terminal position and is linked to the remainder of the molecule through its 5′-hydroxyl group, and is also esterified at C-3′ with phosphoric acid. Structures of this type are consistent with the observations of Schmidt, Cubiles & Thannhauser (1947) who reported that the action of phosphomonoesterase on the products of ribonuclease digestion is to hydrolyse off all the phosphoric acid stable to N-hydrochloric acid at 100° for 1 hr., i.e. that bound to pyrimidine nucleosides, while leaving all the rest of the phosphoric acid bound.

It will be seen that purine nucleotide-purine nucleotide linkages, and purine nucleotide 3′-pyrimidine nucleotide 5′-linkages are all stable to ribonuclease, as well as the 2:3′-cyclic phosphate linkages in cyclic purine nucleotides. The linkages which are broken are those which leave cyclic 2′:3′-

pyrimidine nucleotide residues at the end of a molecule, and this cyclic link is subsequently broken down itself. The existence of pyrimidine dinucleotides in partial digests and their subsequent hydrolysis by ribonuclease to give nucleoside 3′-phosphates through cyclic nucleotides, shows that it is unnecessary to postulate a branching chain to explain the formation of free pyrimidine nucleotides in the enzymic digests. These observations are consistent with the view that ribonuclease will only break secondary phosphate esters of pyrimidine nucleoside 3′-phosphates, that is to say, it is a highly specific phosphodiesterase which will only hydrolyse secondary phosphate esters of pyrimidine ribonucleoside 3′-phosphates. This specificity is reflected in its ability to hydrolyse the cyclic 2′:3′-monohydrogen phosphates of the pyrimidine ribonucleosides, which themselves have this structure.

Cohn (1951) has shown that all the pyrimidine nucleotides released from RNA by ribonuclease action are of the '*b*' form (which we have assumed to be the 3′-phosphates) and Brown, Dekker & Todd (1952) have shown that the end product of the ribonuclease digestion of the nucleoside 2′:3′-cyclic phosphates is also the '*b*' form, a finding which we have confirmed in the case of the cyclic cytidylic acid by running the digest in solvent 2, which separates the '*a*' and '*b*' cytidylic acids in a run of about 70 cm., the '*b*' form having the smaller R_F value. As a consequence of the fact that all the pyrimidine nucleotides found in those polynucleotides which are resistant to further ribonuclease digestion are at the ends of the molecules, it will be seen that they also will have been fixed by the enzyme action as the '*b*' isomers. Consequently, subsequent alkaline hydrolysis of such polynucleotides will only give the '*b*' pyrimidine nucleotides, although both forms of the purine nucleotides will be found.

As all the nucleoside residues in ribonuclease digests are substituted in the 3′ position, none of the substances described, excepting the dinucleoside monophosphates, react with periodate as do the nucleosides and their 5′-phosphate esters. Terminal nucleotide residues dephosphorylated with phosphomonoesterase will, however, react with periodate and this explains some of the observations made by Cavalieri, Kerr & Angelos (1951) and by Schmidt, *et al.* (1947) on RNA digested by both enzymes.

The large number of different polynucleotides obtained in digests of individual specimens of RNA leads one to the conclusion that either RNA is a very large molecule indeed, or that it consists of a large number of small molecules of varying composition. We are inclined to the latter view and in the next paper in this series will give evidence in support of this. In some of these small molecules a large number of pyrimidine nucleotide residues must be adjacent to each other, as a minimum of four is necessary in order to get a trinucleotide of the type of UUU! liberated from the middle of a chain as a result of ribonuclease digestion. It should be possible, if the rates of enzymic hydrolysis of the various internucleotide links were known, to calculate the relative numbers of adjacent pyrimidine nucleotide residues from the quantities of U, U!, UU! etc., and the similar cytidylic acid derivatives using probability theory. Whether this will be of any practical use is at the moment dubious, nor is there much point in devoting much time to a step-by-step degradation process of what is obviously a mixture. While these nucleic acids are clearly complex mixtures, we have evidence that they are not merely random mixtures and this evidence we will also present in the next paper.

It will be appreciated that, as so many polynucleotides exist in ribonuclease digests, the quantities of most of the individual polynucleotides are very small indeed. In fact most of the analyses reported in this paper have been made on a total of about 0·5 mg. of material, which in some cases has been subjected to two or more enzyme digestions and several chromatographic or electrophoretic separations. It is therefore not surprising that the accuracy of the analyses made on these substances is not quite as high as we have obtained on whole nucleic acid, but it will be noted that the accuracy is still of the same order as is obtained by other methods.

SUMMARY

1. The smaller products of ribonuclease digestion of ribonucleic acids have been isolated by a method involving a preliminary chromatographic separation followed by paper electrophoresis.

2. Detailed instructions for the isolation of fifteen of the smaller polynucleotides have been given.

3. The theory of the separation of polynucleotides by paper electrophoresis has been discussed.

4. Several dinucleotides have been subjected to a degradation process which shows that their general structure is: pyrimidine nucleoside 5′-(nucleoside-3′ or 2′-phosphate) ester 3′ or 2′-phosphate, or the cyclic form of such a dinucleotide: pyrimidine nucleoside 5′-(nucleoside-3′ or 2′-phosphate) ester 2′:3′-cyclic phosphate.

5. During ribonuclease digestion the cyclic forms of the dinucleotides are first liberated and the 2′:3′-cyclic phosphate link is subsequently slowly hydrolysed by the enzyme to give the 3′- (or 2′-) phosphates.

6. Trinucleotides liberated by ribonuclease action all have at least one pyrimidine nucleotide residue.

7. The alkali-labile link in ribonucleic acids is at C′-5.

8. Ribonuclease hydrolyses certain esters of pyrimidine ribonucleoside 3′- (or 2′-) phosphates.

9. Four dinucleoside monophosphates have been prepared by hydrolysis with ribonuclease followed by phosphomonoesterase.

10. A structure for ribonucleic acid has been proposed which satisfies these results on the basis of an unbranched chain.

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The Structure of Ribonucleic Acid

3. THE END GROUPS, THE GENERAL STRUCTURE AND THE NATURE OF THE 'CORE'

By R. MARKHAM and J. D. SMITH

Agricultural Research Council, Plant Virus Research Unit, Molteno Institute, University of Cambridge

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Although much is known about the size and the various physical properties of the deoxyribonucleic acids, there is less information about the ribonucleic acids (RNA), largely because they are more difficult to prepare in an undegraded state, and to some extent also because they are easily and cheaply available commercially in a partially degraded form. In addition, RNA from many animal sources is inevitably subjected to enzyme action during isolation. The plant viruses offer what is perhaps the most suitable material for such investigations; some of them will yield nucleic acid under the mildest treatment (Markham & Smith, K. M., 1949). We also have some evidence that it is possible to prepare the RNA of yeast in a relatively undegraded form.

The determination of the molecular weights of such highly charged and asymmetrical molecules as those of the nucleic acids is a matter of extreme difficulty. Several attempts have been made to obtain this information for RNA (e.g. Tennent & Vilbrant, 1943; Bacher & Allen, 1950*a*), but it is likely that the results are only of the correct order of magnitude.

By more direct methods of a chemical nature, one can get an idea of the average molecular complexity. The various titration studies fall in this category (e.g. Fletcher, Gulland & Jordan, 1944), but these were made on partially degraded materials and probably involved some extra degradation as well. In this paper we shall describe how one can determine the average chain length of RNA and hence calculate the number of nucleotide residues per chain.

It has been known for some time that the course of digestion of RNA by ribonuclease stops before all the nucleotides are liberated. Kunitz (1940) found that 40% of his RNA was still precipitated by uranyl acetate after it had been digested to completion by the enzyme, while several workers have reported that a large fraction (about 10–25%) of the digested RNA is unable to dialyse through cellophan film (Bernheimer & Rodbart, 1948; Bacher & Allen, 1950*b*), although the observation of Greenstein, Carter & Chalkley (1947) to the contrary does not seem to have attracted much attention.

The 'non-dialysable' fraction of digested RNA has been called, among other names, the 'core' (e.g. Magasanik & Chargaff, 1951), and we propose to adopt this convention in this paper, although we hope to demonstrate that its non-diffusibility

through cellophan film is largely due to charge effects rather than to the actual size of its constituents. The core is in fact merely a mixture of polynucleotides having a structure similar to that of the ribonuclease-stable di- and tri-nucleotides which we have already described, i.e. a chain of purine nucleotide residues terminated at one end by a pyrimidine nucleotide residue.

The proposed structure of ribonucleic acid

We believe that the general structure of RNA is a series of unbranched chains of varying composition with the general features of the following diagram in which the nucleotide residues are represented by A, G, C and U for adenylic, guanylic, cytidylic and uridylic acid residues respectively:

$$AC:U:C:U:C:C:AGAGC:U:C:C:AAGU:$$
$$U:GU:U:C:C:GC:C:U:AGC:A!$$

The exclamation mark after the terminal A denotes that it is a cyclic nucleotide. Each nucleotide is joined through a phosphate ester link on its C-2′ (or 3′) to the adjacent residue on the right-hand side and through a similar link on C-5′ to its neighbour on the left (this nomenclature is the same as that used in the preceding paper). The bonds which are broken by ribonuclease are represented by colons, and those residues which can then be dephosphorylated by phosphomonoesterase are shown in italic type. Thus it will be seen that the chain shown above will give rise on complete ribonuclease digestion to fourteen pyrimidine mononucleotides, three dinucleotides, one trinucleotide, one tetranucleotide, one pentanucleotide and a cyclic purine nucleotide, and it will be seen that on subsequent phosphomonoesterase treatment all the pyrimidine nucleotide residues will be dephosphorylated. It necessarily follows from our theory of ribonuclease action (Markham & Smith, 1952*b*) that the cyclic purine nucleotide could only be liberated if it were situated at one end of the chain. It must not, however, be thought that all chains are necessarily terminated by cyclic purine nucleotides. Pyrimidine nucleotides are also to be found in this position, and in commercially prepared RNA, where the average chain length has been reduced, probably by treatment with alkali, to only 6–8 units or even less, adenylic acids '*a*' and '*b*' and guanylic acids '*a*' and '*b*' (Carter, 1950) are also to be found at chain ends, as well as AG (Markham & Smith, 1951). The core is composed of the tetra- and penta-nucleotides and possibly some of the trinucleotides.

A nucleic acid chain of the type depicted above is terminated on the left-hand side by a nucleoside residue linked through the hydroxyl group on C-2′ (or 3′) to its neighbour by a phosphate ester link, and on the right by a nucleotide linked to the chain by its 5′-hydroxyl group. Residues of the latter type we shall call terminal groups and these may be determined in the following ways:

(*a*) If they are cyclic purine nucleotides linked to the rest of the chain through a pyrimidine nucleotide residue, the RNA is digested to completion with ribonuclease and the A! and G! isolated chromatographically (it will be noted that in the *iso*propanol-ammonia solvent (solvent 1, Markham & Smith, 1952*a*) these are to be found in bands 5*a* and 6, well ahead of any other possible constituents and consequently very small amounts may be detected in large quantities of digest).

(*b*) If the terminal groups are nucleoside 2′- or 3′-phosphates they may be dephosphorylated by phosphomonoesterase. On subsequent hydrolysis of the RNA with sodium hydroxide these groups will then yield nucleosides while all the others will yield nucleotides. In solvent 1 the latter appear in bands 2 and 4, while the nucleosides run well ahead and may then be separated and identified. Some of these terminal groups may also be determined on ribonuclease digests. For example, a terminal purine nucleoside 2′- (or 3′)-phosphate, if linked to a pyrimidine nucleotide residue, will be liberated as a nucleotide on ribonuclease digestion and may then be separated from the digest and identified (Markham & Smith, 1951).

(*c*) Terminal cyclic pyrimidine nucleotides will not be detected by any of these methods as they are not attacked by phosphomonoesterase, and ribonuclease digestion does not distinguish them from other pyrimidine residues. We know of no method of revealing such end groups.

(*d*) Terminal cyclic purine nucleotides linked through a purine nucleotide residue to the chain will appear in ribonuclease digests as polynucleotides containing only purines. We have not attempted the identification of such substances as they must be present in very small quantities.

Another aspect of structure which is of interest is the distribution of the various nucleotides in the polynucleotides which are found in ribonuclease digests. The quantitative isolation of analogous pairs of substances such as AU and AC, or GU and GC, or AU and GU gives a measure of the frequencies with which individual nucleotides occur in certain possible positions in the nucleic acid chain. In this way we have shown for two different RNA's that there is a certain amount of order in the arrangement of the purine nucleotides in the chains.

METHODS AND MATERIALS

The methods and materials used were essentially the same as those described in the previous papers in this series (Markham & Smith, 1952*a*, *b*). For convenience we shall list again the various chromatographic systems used: (1) 70% *iso*propanol-water (v/v) with NH_3 in the vapour

phase; (2) sat. $(NH_4)_2SO_4$, 80 parts, *iso*propanol, 2 parts; 0·5M-sodium acetate, 18 parts (v/v/v); (3) *n*-butanol saturated with water, 90 parts; formic acid, 10 parts (v/v); (4) *iso*propanol, 170 ml.; conc. HCl, 44 ml.; water to 250 ml. Solvent 1 separates the products of ribonuclease digestion into a number of bands which we have numbered 1 to 6 in order of R_F values. The details of this separation and of the paper electrophoresis are to be found in the previous papers.

Digestion of RNA with ribonuclease

In all experiments to be described digestion with ribonuclease was carried to completion. Hydrolysis was at 55° usually with 0·1 mg. ribonuclease/ml. and constantly adjusted to pH 7·6, until chromatographic analysis showed that all the cyclic pyrimidine nucleotides had been hydrolysed. The hydrolysis of the latter by ribonuclease proceeds much more slowly than the breakdown of the internucleotide linkages so that the disappearance of cyclic pyrimidine nucleotides marks the end of ribonuclease digestion.

Isolation of the 'core'

RNA completely digested with ribonuclease was dialysed in cellophan sacs against distilled water with stirring for about 48 hr. at which time the rate of loss of material from the sac was extremely small. Samples of such cores were usually treated again with ribonuclease and examined chromatographically to ensure that no undegraded RNA remained.

The isolation and estimation of purine nucleotides in ribonuclease digests

A ribonuclease digest of RNA (8–16 mg.) was placed in a line across the top of a sheet of filter paper (Whatman no. 3) and developed in solvent 1. A! and G! were found in bands 6 and 5*a*, and their identification has already been described (Markham & Smith, 1952*a*). The bands were cut out, eluted, concentrated and run as a chromatogram in solvent 2. The spots of A! and G! were eluted and estimated spectrophotometrically.

Adenylic acids '*a*' and '*b*' were found in band 4 of the chromatogram using solvent 1, together with the pyrimidine nucleotides, and were separated from the latter by running in solvent 2. Guanylic acids '*a*' and '*b*' were in band 2 together with AC, AU and certain trinucleotides. They were isolated using solvent 2, in some cases after a preliminary fractionation by paper electrophoresis. The adenylic and guanylic acids were identified by chromatography in solvents 1, 2 and 4, by their ultraviolet light absorption spectra and by their movement on paper electrophoresis at pH 3·5. On hydrolysis with N-HCl for 1 hr. at 100° they were quantitatively converted to adenine and guanine respectively.

The estimation of end groups in RNA and the 'core' by phosphomonoesterase treatment

The intact RNA and the core were incubated with purified prostate phosphomonoesterase (Markham & Smith, 1952*b*) at 37° in 0·1M-acetate buffer, pH 5, for sufficient time to ensure the complete liberation of the terminal phosphoryl groups. This time was estimated from the rate of dephosphorylation of solutions of pure adenylic and guanylic acids by the enzyme preparation, and under these conditions the rate of dephosphorylation of cyclic nucleotides was negligible. On further incubation of the digests no more phosphate was liberated and chromatograms in solvent 2 showed no evidence of phosphodiesterase or of ribonuclease activity (although by using a much greater enzyme concentration and incubating at pH 7·5 and 55°, it was possible to demonstrate that the enzyme did contain a small amount of ribonuclease). NaOH was then added to give a concentration of about 0·3N, and the digest incubated at 37° for 4–5 hr. when all the internucleotide links were hydrolysed to give mononucleotides plus the nucleosides derived from the dephosphorylated terminal groups. Intact RNA treated in this way with NaOH gave no nucleosides.

The nucleosides were isolated from these digests by neutralizing them with acetic acid, and chromatographing the solution in solvent 1 on Whatman no. 3 paper. In this solvent the nucleosides were to be found in two bands moving ahead of the nucleotides. The slower of the two contained guanosine, and the other adenosine, cytidine and uridine. The guanosine was further purified by chromatography in solvent 2. This solvent also separated adenosine from the mixture of adenosine, cytidine and uridine, while solvent 3 resolved cytidine from adenosine and uridine. Uridine could thus be estimated by difference. All estimations were made spectrophotometrically.

RESULTS

The end groups of RNA

Certain of the terminal phosphorylated groups have been identified and quantitatively estimated in two ribonucleic acids, that prepared from yeast by a mild method (YNA 1) and the nucleic acid isolated from turnip yellow mosaic virus at neutral pH (Markham & Smith, 1952*b*). The end groups estimated comprise the purine nucleotides (A!, G! and the adenosine and guanosine 2′- and 3′-phosphates) linked to the nucleic acid chain through a pyrimidine nucleotide residue, all of which were isolated from ribonuclease digests, and all non-cyclic purine and pyrimidine end groups which were isolated as nucleosides after treatment of the nucleic acid with phosphomonoesterase and alkali. It will be appreciated that, in the estimation of the latter type of end group, contamination of the phosphomonoesterase preparations with ribonuclease could give spuriously high values for pyrimidine nucleotide end groups, while the presence of diesterase would affect the values for both purine and pyrimidine nucleotides. The control experiments described earlier eliminated these possibilities.

Table 1 lists the types of terminal groups found and their relative abundance in the two nucleic acids. Some of these occur in very small amounts and we would not claim a high degree of precision in their estimation. Remembering that certain types of phosphorylated end groups are excluded from this list, and that the purine nucleoside 2′- and 3′-phosphates estimated using phosphomonoesterase include the end groups of this type also found in ribonuclease digests, it is possible to calculate

Table 1. *The terminal groups of ribonucleic acids of yeast and the turnip yellow mosaic virus*

(Amounts are in moles/100 moles of nucleotide residues in the intact nucleic acids. The colon denotes a bond which is broken by ribonuclease, and italic type shows that the end group is attacked by phosphomonoesterase.)

End group	Method of determination	Turnip yellow mosaic virus nucleic acid	Yeast nucleic acid
:A!	Complete ribonuclease digestion	0·16	0·88
:G!	Complete ribonuclease digestion	0·20	1·16
:*A*	Complete ribonuclease digestion	None detected	<0·58
:*G*	Complete ribonuclease digestion	None detected	<0·26
A	Phosphomonoesterase + NaOH hydrolysis	0·17	1·06
G	Phosphomonoesterase + NaOH hydrolysis	0·18	1·40
C	Phosphomonoesterase + NaOH hydrolysis	0·97	1·81
U	Phosphomonoesterase + NaOH hydrolysis	0·22	1·90

maximum mean chain lengths for the two nucleic acids. These are twelve and fifty-three residues for the yeast and virus nucleic acids respectively.

The distribution of nucleotides among the ribonuclease-resistant digestion products

The mononucleotides, dinucleotides and certain of the trinucleotides were quantitatively isolated from complete ribonuclease digests of the two nucleic acids by methods we have described in detail elsewhere (Markham & Smith, 1952*a*, *b*). Each polynucleotide was analysed for its component purines and pyrimidines. The pyrimidine nucleotides were found in band 4 of chromatograms run in solvent 1 together with adenylic acid if present. The absence of other components in this band (shown by chromatography in solvent 2 and by paper electrophoresis) made it possible to estimate the free cytidylic and uridylic acids by chromatography in solvent 4 after hydrolysis for 1 hr. at 100° in N-HCl which converts the adenylic acid to adenine.

YNA (1) and the 'cores' from turnip-yellow-mosaic-virus nucleic acid and a second preparation of a yeast ribonucleic acid (YNA 2) were analysed for their purines and pyrimidines (Table 2). It should be noted that YNA (1) and YNA (2) differed in composition. (We have noted this for several preparations.)

In Tables 3 and 4 the results are arranged to show the distribution of the two pyrimidine nucleotides among the digestion products, and Table 5 gives similar data for adenylic and guanylic acids.

Both nucleic acids have the following features in common: (*a*) About 30–45 % of the uridylic and cytidylic acid residues are liberated in complete digests as the free nucleotides, while 3–5 % appear as AC and AU. A considerable proportion of the nucleotides is present in polynucleotides containing three to five residues which have not yet been isolated individually.

(*b*) The ratios of the two pyrimidine nucleotides present in pairs of analogous substances such as C and U, GC and GU, and in the 'core' are in all cases very close to those found in the whole nucleic acids.

Table 2. *Molar ratios of the nucleotides (to an arbitrary total of 4) of the nucleic acids of yeast and the turnip yellow mosaic virus*

	Yeast ribonucleic acid (1)	Core from yeast RNA (2)	Turnip yellow mosaic virus RNA	Core from turnip yellow mosaic virus RNA
Adenylic acid	1·15	1·32	0·91	1·42
Guanylic acid	0·97	1·62	0·69	1·34
Cytidylic acid	0·78	0·49	1·53	0·75
Uridylic acid	1·10	0·56	0·89	0·49

(*c*) The proportions of the two purine nucleotides found in the dinucleotide fraction, the 'core' and the cyclic purine nucleotides differ from each other and from those of the whole nucleic acid.

The 'core' of RNA

'Cores' prepared from the ribonucleic acids of yeast (YNA 2) and turnip yellow mosaic virus were analysed for the bases (Table 2) and contained respectively 26 and 31 % of the bases as pyrimidines (on a molar basis).

The terminal phosphorylated groups of the YNA 'core' were identified in the following way. The 'core' was treated with phosphomonoesterase to dephosphorylate the terminal groups and the digest hydrolysed with sodium hydroxide. Terminal residues were thus converted to nucleosides and other residues to nucleotides. The alkali digest was neutralized with acetic acid and run on a two-dimensional chromatogram in solvents 1 and 2. Pyrimidine nucleosides and the '*a*' and '*b*' forms of adenylic and guanylic acids were the only substances found. It follows that all the pyrimidine nucleotides

Table 3. *The distribution of cytidylic and uridylic acid among some of the products of the complete ribonuclease digestion of yeast RNA* (1)

(All values are in moles/100 moles of nucleotide residues in the intact RNA.)

Substance	Cytidylic acid present	Substance	Uridylic acid present	Molar ratio uridylic acid/ cytidylic acid
C	5·63	U	8·41	1·49
AC	0·77	AU	1·02	1·32
Whole RNA	19·5	Whole RNA	27·5	1·41

Table 4. *The distribution of cytidylic and uridylic acid among some of the products of the complete ribonuclease digestion of turnip-yellow-mosaic-virus nucleic acid*

(All values are in moles/100 moles of nucleotide residues.)

Substance	Cytidylic acid present	Substance	Uridylic acid present	Molar ratio uridylic acid/ cytidylic acid
C	15·3	U	9·6	0·63
AC	1·86	AU	1·06	0·57
GC	0·67	GU	0·43	0·65
AAC	0·25	AAU	0·15	0·60
The 'core'	4·02	The 'core'	2·64	0·65
Average ratio	—	—	—	0·62±0·035
Whole nucleic acid	38·3	Whole nucleic acid	22·3	0·58

in the 'core' are in a terminal position and these are the only terminal groups. The polynucleotides in the 'core' thus have the structure common to all the ribonuclease-resistant polynucleotides, that is a chain of purine nucleotide residues terminated by a pyrimidine nucleotide. With this knowledge one may calculate the mean chain length from the proportions of pyrimidine nucleotides in the 'core'. For the virus nucleic acid 'core' this is 3·2 residues and for YNA (2) 'core' 3·9 residues.

Table 5. *The distribution of the two purines in fractions of complete ribonuclease digests of yeast ribonucleic acid* (1) *and turnip-yellow-mosaic-virus nucleic acid*

Ratio	Yeast ribonucleic acid (1)	Turnip yellow mosaic virus nucleic acid
A/G in whole nucleic acid	1·18	1·32
A/G in 'core'	—	1·06
A!/G!	0·75	0·81
AC/GC	—	2·78
AU/GU	—	2·44

Dialysis of the 'core'

If the 'core' is placed in a cellophan sac and dialysed against water the polynucleotides diffuse out extremely slowly. The isolation of 'core' of course depends on this fact, and these 'cores' have already been dialysed against water for considerable periods. If, however, the water outside the sac is replaced by 2M-sodium chloride the rate of loss of polynucleotides from the sac is greatly increased and with rapidly stirred dialysis against changes of concentrated sodium chloride solution almost all the material passed from the sac in a few hours.

DISCUSSION

In the first two papers of this series (Markham & Smith, 1952*a*, *b*) we have shown that the specificity of ribonuclease is such that it breaks ester linkages of pyrimidine nucleoside 2′- (or 3′)-phosphates joining adjacent ribose residues presumably by a 2′:5′-internucleotide link,* to give rise to mono-, di- and tri-nucleotides, and that there is no reason to postulate a branched structure to explain the formation of these substances. The only other position in which branching could occur is in the so-called 'core'. Now this core has quite definite chemical properties which fix limits on its structure. One in every three or four residues on the average is terminal and bears a secondary phosphoric acid —OH group. We have shown, in agreement with the findings of Schmidt *et al.* (1951), that the latter is always associated with pyrimidine nucleotide residues. The whole core is hydrolysed by alkali to give nucleoside 2′- or 3′-phosphates (or in the case of the purine nucleotides, both the '*a*' and the '*b*' forms) so that the 2′ or 3′ group in each residue is phosphorylated. It is difficult to visualize a chain branching on sugar residues which could have all these properties, bearing in mind that any postulated structure must allow the formation of 2′:3′ cyclic phosphates to account for the alkali lability of the internucleotide link. Fortunately it is not necessary to postulate any branching to explain the existence

* In the two preceding papers we have assumed that the link is 3′-5′ by analogy with deoxyribonucleic acid. Experiments which we have since done on the latter make us suspect that if the two structures had this similarity deoxyribonuclease would digest RNA.

of the core, for, as we have shown, it can diffuse quite readily through cellophan film provided that sufficient salt is present in the solution. Because of this there is no reason to suppose that it is anything other than a mixture of short chains, about three or four purine nucleotide residues in length, terminated by a pyrimidine nucleoside 2′- (or 3′)-phosphate joined to the rest of the chain by its 5′-hydroxyl group. That is to say the core is only a mixture of polynucleotides having the same general properties as the ribonuclease-stable di- and tri-nucleotides. In the absence of salts these polynucleotides associate or are prevented by charge effects from dialysing, while in the presence of salts these relatively small molecules can diffuse quite readily through cellophan film. This explains the observations of Greenstein *et al.* (1947) on the (apparent) activating effect of sodium chloride on the rate of ribonuclease action, and also suggests that their nucleic acid was badly degraded even before they added the enzyme. The non-dialysability in distilled water of polynucleotides is not surprising as they are covered with charged groups. The final picture of RNA would seem to be an unbranched chain.

We have attempted to measure the length of the RNA chains by revealing their end groups. While we do not claim great precision for the estimate of the chain lengths which we have made, especially as we cannot hope to reveal those ends which consist of cyclic pyrimidine nucleotide residues, it is obvious from the results that the chains are quite short and of many kinds. This conclusion follows not only from the frequency of the observed end groups, but also from the relative abundance of the large number of polynucleotides which we have described in the previous papers, while there must be several trinucleotides as yet unidentified, not to mention the tetra- and penta-nucleotides comprising much of the core.

The distribution of the pyrimidine nucleotides among the various mono-, di- and poly-nucleotides of the two different types of RNA which we have investigated shows a remarkable consistency which gives some indication of the structural arrangement in the chains. The end products of ribonuclease digestion, with the exception of terminal groups, are all of a type which can be represented by the formulae Pu*C* and Pu*U*, in which Pu is n purine nucleotide residues ($n \geqslant 0$; if $n=0$, Pu*U* is free uridylic acid). For any pair Pu*C* and Pu*U* having the Pu part of the molecules identical, we have found that the molar ratio Pu*C*/Pu*U* has a value approximating closely to the ratio of cytidylic acid to uridylic acid in the intact nucleic acid and in the 'cores'. These ratios are shown for some of the polynucleotides in the two RNA's in Tables 3 and 4. Bearing in mind the specificity of ribonuclease action, the occurrence of a pyrimidine nucleotide residue in a substance such as Pu*C* or Pu*U* fixes to some extent its position in the intact nucleic acid. Thus the cytidylic acid residue in Pu*C* can only occupy one of the positions shown as (*a*), (*b*) and (*c*) (here use has been made of the notation introduced earlier in this paper, while R represents any residue and *X* a pyrimidine nucleotide)

X:Pu*C*:R	*X*:Pu*C*	Pu*C*:R
(*a*)	(*b*)	(*c*)

and similarly for Pu*U*. In the structures of this type *U* and *C* would appear to be interchangeable. This materially reduces the amount of isomerism possible in the nucleic acid chains. The distribution of the purine nucleotide residues, on the other hand, is of a different type as the ratios of A/G in the core, the dinucleotide fraction and the intact RNA's differ widely from each other (Table 5).

SUMMARY

1. Two methods have been developed for the identification of nucleotides which occupy end positions in ribonucleic acid chains and bear terminal phosphoryl groups.

2. The cyclic nucleotides adenosine 2′:3′-monohydrogen phosphate and guanosine 2′:3′-monohydrogen phosphate, and adenylic, guanylic, cytidylic and uridylic acids have been recognized as end groups in the ribonucleic acids of yeast and turnip yellow mosaic virus.

3. Estimates have been made of the proportions of these terminal groups present in the two nucleic acids. It is concluded that ribonucleic acid consists of mixtures of many kinds of comparatively short chains.

4. The frequencies with which the nucleotides occur among the ribonuclease-resistant digestion products has been studied. The distribution of cytidylic and uridylic acids among the positions occupied by the pyrimidine nucleotides in the intact nucleic acid chain appears to be random. That of guanylic and adenylic acids, however, is not random.

5. The 'core' of ribonucleic acids, that is the fraction of the ribonuclease digest which does not dialyse into water through cellophan film, has been shown to be readily dialysable against salt solutions. It is a mixture of polynucleotides about three to five residues in length and each polynucleotide is a chain of purine nucleotides terminated by a pyrimidine nucleotide residue, with the terminal phosphoryl group on carbon atom 2′ or 3′. The components of the core have the same structure as the other ribonuclease-resistant polynucleotides.

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The *Orobanche* Germination Factor

3. CONCENTRATION OF THE FACTOR BY COUNTER-CURRENT DISTRIBUTION

By R. BROWN, A. D. GREENWOOD, A. W. JOHNSON, A. R. LANSDOWN, A. G. LONG and N. SUNDERLAND
Botany Department, University of Leeds, and Chemical Laboratory, University of Cambridge

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The chromatographic purification of crude concentrates of the *Orobanche* germination stimulant, particularly partition chromatography on cellulose, has been described in an earlier paper (Brown, Greenwood, Johnson, Long & Tyler, 1951*b*). The most active preparations thus obtained had activity values (Brown, Greenwood, Johnson & Long, 1951*a*) of $1–2 \times 10^5$, and it seemed possible that they contained a high percentage of the pure stimulant. However, as these preparations were invariably resinous and as no convenient physical property of the stimulant was known by which its purity could be gauged, other methods of purification have been investigated to ascertain whether concentrates could be obtained with even higher activity values and possibly in a crystalline form.

Much of the present paper is concerned with purification of the *Orobanche* germination factor by partitioning between immiscible solvents, the choice of solvents being limited on the basis of earlier stability studies (Brown *et al.* 1951*b*) to those boiling below 120° and neither strongly basic nor acidic.

In our earlier studies it was shown that the stimulant adsorbed on charcoal could best be eluted with chloroform-methanol and it has now been found that considerable purification of these crude eluates can be achieved by a further extraction of their aqueous solutions with ethyl acetate. This step has been included in all of our later purifications. Ethyl acetate was slightly superior to chloroform or ether for this extraction step.

The partition method of purification was extended to the use of counter-current methods, and many solvent systems were tried before a satisfactory distribution ratio of the active stimulant was achieved between the two immiscible phases. Some idea of the partition coefficient of a compound between heterogeneous solvent phases can be obtained from preliminary paper-strip chromatograms (Brown *et al.* 1951*b*). The relationship of the R_F value in partition chromatography to the partition coefficient has been discussed by Martin & Synge (1941), by Consden, Gordon & Martin (1944) and by Craig (1950).

The mixture *tert.*-butanol (4)–light petroleum (b.p. 60–80°; 5)–acetone (4)–water (2) was eventually devised and this gave an appreciable concentration of the active material in the centre tubes of the counter-current apparatus, i.e. the partition was approximately equal between the two phases. *tert.*-Butanol was selected as the alcoholic component in order to cut down any esterification of lactone or acid groupings believed to be present in the molecule of the germination factor. The use of buffered solutions in the counter-current extractions to suppress such undesirable changes seemed inadvisable in view of the instability of the factor in alkaline or strongly acid solutions and the impossibility of carrying out the biological test in the presence of buffer solutions of the usual concentrations.

The purified stimulant obtained either by chromatography (Brown *et al.* 1951*b*) or by extraction methods was only slightly soluble in water, and

for testing purposes the product was dissolved in a little methanol and a large volume of water added. The methanol was then removed under reduced pressure and the resulting emulsion used for the assay. Such emulsions could not be separated in the centrifuge so that fractional precipitation offered little promise as a means of purification.

The progress of isolation and purification of a biologically active material is determined to a large extent by the speed and accuracy of the biological testing. In this case the results of any particular test were not available before 7 days and consequently characteristic physical properties of the active material were sought in order that the various fractions might be assessed more rapidly.

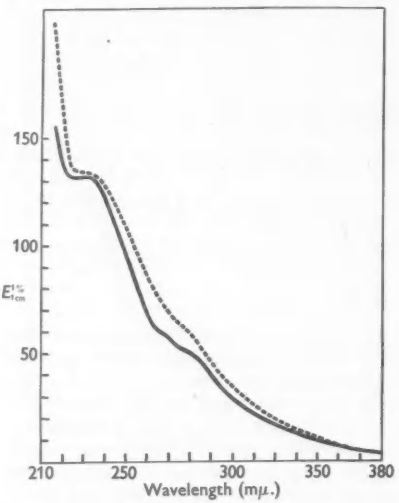

Fig. 1. Ultraviolet absorption spectrum of the crude *Orobanche* germination stimulant (activity value, 63 000). ——, ethanol solution; - - - - -, aqueous solution.

The ultraviolet absorption spectrum (Fig. 1) of the crude concentrate before counter-current purification or even after distribution between chloroform and water did not reveal any characteristic features other than an arrest at 2300–2350 A. and a slight inflexion at 2800 A. However, after purification by the four-component system, *tert.*-butanol–petroleum (b.p. 60–80°)–acetone–water as described above, the ultraviolet absorption spectrum of the most active fraction exhibited a distinct maximum at 2450 A. ($E^{1\%}_{1\,cm.} = 170$) (Fig. 2), and it seems probable that this ultraviolet absorption is a feature of the biologically active material.

Like the *Striga* germination factor (Brown, Johnson, Robinson & Tyler, 1952) the *Orobanche* factor can be distilled under high vacuum. Fig. 4 shows the results of a typical fractionation, and it will be seen that most of the active material distils at temperatures around 100° at a pressure of 10^{-5} mm. Although considerable loss of total activity is incurred in these distillations, the fact that the stimulant can be distilled at all is clear indication that the anhydrous concentrate is much more stable than its aqueous solutions. The ultraviolet absorption spectrum of the biologically active fraction of the distillate again showed a distinct maximum at 2450 A. ($E^{1\%}_{1\,cm.} = 160$).

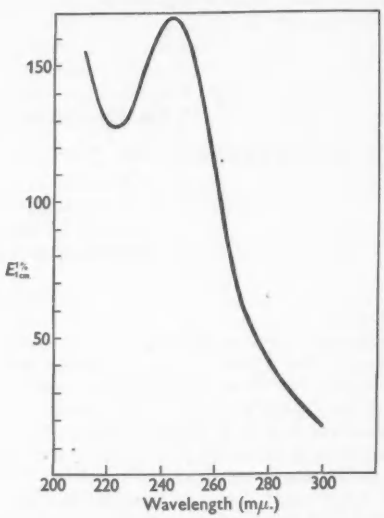

Fig. 2. Ultraviolet absorption spectrum of purified *Orobanche* germination stimulant (activity value, 100 000) in ethanol.

EXPERIMENTAL

Concentration of the germination factor by solvent extraction of the crude concentrates. The crude eluate (83·7 mg.) obtained from the charcoal (Brown *et al.* 1951*a*) was dissolved in water (20 ml.; activity of solution, 10 600) and extracted with ethyl acetate (5 × 100 ml.). The solvent was removed from the combined extracts below 40° or preferably by freeze-drying and the water-soluble fraction (6·5 mg.) of the residue (16·2 mg.) tested for activity (122 000; recovered activity, 90%). Similar experiments were carried out using $CHCl_3$ (85–89% activity recovery) and diethyl ether (75% activity recovery).

Counter-current extraction of concentrates of the germination factor. The material used for the counter-current extractions was obtained by eluting the charcoal with $CHCl_3$-methanol (Brown *et al.* 1951*a*) and then extracting an aqueous solution of the product with ethyl acetate as described above. A 25-tube counter-current apparatus (Craig & Post, 1949) was used, and after the distribution the combined phases from each tube were evaporated to dryness, the efficiency of

the separation being assessed by determinations of the weight, optical density and biological activity of each fraction. Results using the 4-component, 2-phase system, water (2)–petroleum (b.p. 60–80°; 5)–*tert.*-butanol (4)–acetone (4) are expressed in Fig. 3.

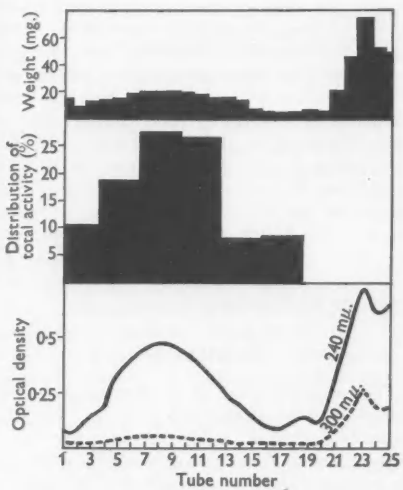

Fig. 3. Counter-current distribution of a concentrate of the *Orobanche* germination factor between *tert.*-butanol–petroleum (b.p. 60–80°)–acetone–water.

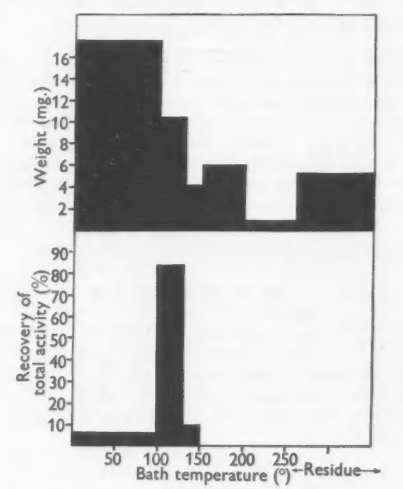

Fig. 4. Distillation of a concentrate of the *Orobanche* germination factor at 10^{-5} mm.

Distillation of the germination factor. The crude concentrate (38·4 mg.; activity 5600) was subjected to high vacuum distillation at 1×10^{-5} mm. with a very short path length, and several fractions were taken off on a cold finger, the bath temperature being raised at the rate of 20°/hr. The weight and activity of each fraction were determined and the results are expressed in Fig. 4.

Titration of the germination factor. The concentrate (18 mg.; activity, 67 000) was dissolved in 0·1 N-tetramethylammonium hydroxide (3·0 ml.) and kept for 2 days at room temperature with exclusion of CO_2. The solution was agitated by the passage of a stream of purified N_2 through it and titrated against 0·2 N-HCl in an electrometric apparatus with a glass electrode. From the amount of acid (0·89 ml.) required to neutralize the excess alkali, the equivalent weight was calculated to be 148.

In a further experiment the same concentrate (18 mg.) was dissolved in 0·1 N-NaOH and the solution heated at 100° for 10 min., cooled and titrated as before. This experiment gave a value of 133 for the equivalent weight. In each case the pH of the alkaline solution of the concentrate decreased on keeping.

DISCUSSION

Counter-current extraction is a useful method of purification in the case of the *Orobanche* germination stimulant, the most active material thus produced having an activity value of the same order (250 000) as that obtained by partition chromatography on columns of cellulose (Brown *et al.* 1951*b*), and it seems reasonable to assume that both materials contain an appreciable amount of the pure stimulant. From the experimental point of view the counter-current method is slightly easier to carry out than the chromatographic method and has been adopted in most of the recent work. The preferred method of purification of the concentrate at the present time therefore comprises (i) adsorption on charcoal, (ii) elution with chloroform-methanol, (iii) extraction of an aqueous solution of the product with ethyl acetate or, better, washing an ethyl acetate solution with water, (iv) counter-current distribution between chloroform and water, (v) counter-current distribution using the *tert.*-butanol–petroleum (b.p. 60–80°)–acetone–water mixture.

The fractional distillation of the concentrates under high vacuum with a short path length also has resulted in a marked concentration of the factor in one fraction. This is a somewhat surprising result in view of the instability to heat of solutions of the concentrates (Brown *et al.* 1951*a*) in polar solvents, especially water, and it therefore seems that the solvents accelerate deactivation. The rate of deactivation of slightly alkaline aqueous solutions of the factor seemed to be independent of the presence or absence of oxygen.

Only preliminary evidence is available on the chemical nature of the *Orobanche* germination factor at the present time. Moreover, in the absence of the pure factor, the necessity of working only with non-crystalline concentrates makes it very difficult to differentiate between the properties of the factor

itself and those of any impurities which might be present. However, on the basis of the comparable activity values of purified concentrates obtained by partition chromatography or by counter-current extraction, the products have been used for preliminary degradative work. Sulphur, phosphorus and halogens were absent, and the small nitrogen values (about 0·5%) obtained in several analyses are unlikely to be significant. Carbon and hydrogen determinations on various concentrates (activity, $1–2 \times 10^5$) have given values of C, 59·5–62·0; H, 7·6–8·7% ($C_{10}H_{18}O_4$ requires C, 59·4; H, 8·9%; $C_{11}H_{16}O_4$ requires C, 62·3; H, 7·6%) after drying at a pressure of 10^{-2} mm. and room temperature over phosphorus pentoxide overnight.

The concentrates are unreactive to Fehling solution, periodate, Schiff reagent and Millon reagent and do not decolorize bromine water. On the other hand, they reduce Tollens reagent and instantly decolorize neutral permanganate in the cold. They are optically inactive (in one case a solution of a sample of concentrate (56 mg.) in chloroform (3 ml.) gave $[\alpha]_D + 1·1° \pm 0·6°$ but generally the rotation was imperceptible), give no colour with ferric chloride and only a slight yellow precipitate with 2:4-dinitrophenylhydrazine. Evidence for a lactone, ester or other potential acid group comes from a positive hydroxamate colour reaction (Feigl, 1947) and the marked fall in pH on keeping freshly prepared alkaline solutions of the factor. The infrared spectrum of a purified concentrate (activity, 1×10^5) gives evidence of a hydroxyl group (band at 2·92 μ.), two ester (or δ-lactone) groups (bands at 5·77 and 5·82 μ.) and also a methyl group (band at 7·27 μ.) and a double bond (band at 6·04 μ.).

It is evident that the *Orobanche* germination factor has some features in common with the *Striga* germination factor described in another series of papers (Brown, Johnson, Robinson & Todd, 1949; Brown *et al.* 1952). This is apparent from the closely similar methods of extraction applicable in each case, and from the closely related physical and chemical properties of each of the concentrates. This supposition has been supported by the results of testing the effect of the *Orobanche* germination factor on *Striga* seeds and vice versa when definite germination effects were obtained in each case. It is also possible that these germination factors may bear some relation to the potato eel-worm hatching factor already examined by Todd and his colleagues (Calam, Raistrick & Todd, 1949 and succeeding papers) although active concentrates of the latter showed little effect on *Orobanche* or *Striga* seeds. The evidence for the free carboxyl group in the eel-worm hatching factor is more definite than in the plant germination factors. Nevertheless, any relationship between these compounds would be of far-reaching biological significance.

SUMMARY

1. The purification of concentrates of the *Orobanche* germination factor using extraction techniques, including counter-current methods, is described. The most active concentrates so obtained have activity values comparable to those obtained by partition chromatography.

2. The germination factor can be distilled at very low pressures.

3. Some chemical properties of the purified concentrates are described.

We are greatly indebted to Prof. A. R. Todd, F.R.S., for his interest and advice throughout this work. We also thank the Agricultural Research Council for supporting this project, and Glaxo Laboratories Ltd., and the Department of Scientific and Industrial Research for grants to A.G.L. and A.R.L. respectively. The determination of the infrared spectrum was carried out by Dr R.N. Haszeldine to whom we accord our thanks.

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The Formation and Breakdown of Pentosephosphates by Liver Fractions

By GERTRUDE E. GLOCK
Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, London, W. 1

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In a previous paper (Dickens & Glock, 1951) it was shown that at least two liver factors are necessary for the oxidation of D-ribose-5-phosphate, one being a coenzyme II-(triphosphopyridine nucleotide; TPN)-specific dehydrogenase and the other an aldolase. Two alternative mechanisms of oxidation were suggested. The first involves an aldolase split into triosephosphate and a C_2 fragment, synthesis of hexosemonophosphate from two C_3 molecules and subsequent oxidation through the appropriate TPN-specific system. The second alternative is the direct oxidation of D-ribose-5-phosphate to 5-phospho-D-ribonic acid followed by oxidative decarboxylation as suggested by Dickens (1936, 1938) for yeast. It has now been shown that this direct oxidative pathway is unlikely to occur, at least in liver. Synthesis of hexosemonophosphate, already demonstrated by Dische (1938, 1949) with haemolysed human erythrocytes and by Schlenk & Waldvogel (1947) and Waldvogel & Schlenk (1947, 1949) with rabbit-liver preparations, has been found to occur very rapidly and to such an extent as to exclude its formation solely from triosephosphate derived from breakdown of pentosephosphate.

MATERIALS

Coenzyme II (TPN), containing 34·5% TPN and 0·6% coenzyme I (DPN), and the barium salts of 6-phospho-D-gluconate (6-PG), synthetic D-glucose-6-phosphate (G-6-P), fructose-6-phosphate (F-6-P) and D-arabinose-5-phosphate were preparations used previously (Dickens & Glock, 1951).

D-*Ribose-5-phosphate* (R-5-P). Two preparations of the barium salt were used. One was prepared by Prof. F. Dickens from barium inosinate according to Levene & Jacobs (1911). (Found: organic P, 8·50; inorganic P, 0·21; pentose (orcinol reaction) 40·6. Calc. for $C_5H_9O_4.PO_4Ba$: P, 8·48; pentose, 41·1%.) This preparation was found to contain some ketopentose. The other preparation was synthesized by Dr C. T. Beer according to the method of Michelson & Todd (1949). (Found: organic P, 8·48; inorganic P, 0·08; pentose, 41·5%.)

5-*Phospho*-D-*ribonate*. The barium salt was prepared by Prof. F. Dickens from R-5-P by Br_2 oxidation.

Ribulose-5-phosphate. This was an equilibrium mixture of the barium salts of ribulose-5-phosphate and ribose-5-phosphate (containing respectively 65 and 35% of these two phosphates) kindly donated by Dr B. L. Horecker.

Mannoketoheptose, *mannosemonophosphate*, *galactose-6-phosphate* and *trehalosemonophosphate*. These were given by Dr Marjorie Macfarlane from the collection of the late Prof. R. Robison.

D-iso*Citrate*. This was generously supplied by Prof. H. A. Krebs in the form of the dimethyl-D-*iso*citrate lactone. Hydrolysis of the lactone and conversion to the sodium salt was effected by incubating 8·08 mg. lactone in 1 ml. 0·02 N-NaOH for 1 hr. at 37° and then diluting with 1 ml. water to give a 0·02 M solution of the sodium salt.

Glycolaldehyde. This was prepared by Miss D. Salmony from dihydroxymaleic acid according to Fischer & Taube (1927).

Crystalline muscle aldolase, triosephosphate and 'Zwischenferment'. These were prepared by Dr P. McLean. Aldolase was prepared according to Cori, Slein & Cori (1947), 0·5 mg. enzyme liberating 24 μg. alkali-labile P in 5 min. at 30° from 1 ml. 0·001 M-hexosediphosphate. Triosephosphate (equilibrium mixture of the barium salts of glyceraldehyde-3-phosphate, and dihydroxyacetone phosphate) was prepared according to Meyerhof (1938), using crystalline muscle aldolase instead of crude muscle extract. Zwischenferment was made from bottom yeast (Anheuser-Busch) according to Kornberg (1950). This preparation, in the presence of TPN, oxidized G-6-P and F-6-P at the same rate (cf. Seegmiller & Horecker, 1951), but was completely inactive towards mannosemonophosphate, galactose-6-phosphate, trehalosemonophosphate, R-5-P, glycolaldehyde and 6-PG (after allowing for its small F-6-P content).

METHODS

Preparation of liver fractions. These were dialysed ammonium sulphate-precipitated horse-liver fractions prepared as previously described (Dickens & Glock, 1951). Fractions *O*, *A*, *B* and *C* are precipitated by 0·3–0·4, 0·4–0·5, 0·5–0·6 and 0·6–0·7 saturation with $(NH_4)_2SO_4$, respectively.

Most of the experiments described in this paper were carried out on these liver fractions prepared from the same horse-liver homogenate. For the assay of hexosemonophosphate an additional preparation of fraction *A* was used since this preparation was completely inactive in oxidizing R-5-P.

Dehydrogenase activity. This was determined either by manometric estimation of O_2 uptake with *N*-methylphenazinium methyl sulphate as carrier or by spectrophotometric measurement of the rate of reduction of TPN at 340 mμ. (see Dickens & Glock, 1951).

Breakdown of ribose-5-phosphate. This was usually followed at 37° in a system consisting of 0·25 ml. 0·05 M-R-5-P, 0·25 ml. 0·25 M-sodium phosphate buffer (pH 7·4), 1 ml. liver fraction and 1 ml. water. At intervals, 0·5 ml. was introduced into 4·5 ml. 5% (w/v) trichloroacetic acid or 4·0% $HClO_4$, and samples of the supernatant used for estimating pentose, glycolaldehyde, etc. Essentially the same results

were obtained when veronal acetate buffer (Michaelis, 1931) or glycylglycine buffer were used.

Hexosemonophosphate formation. After determining pentose disappearance on incubating R-5-P with liver fraction *B* (see above), hexosemonophosphate was estimated in three ways. (*a*) O_2 uptake. After inactivation by boiling, O_2 uptake was determined at 37° with *N*-methylphenazinium methyl sulphate as carrier, on addition of 0·2 mg. TPN and 1 ml. liver fraction *A* (inactive in oxidizing R-5-P, but very active towards G-6-P) and compared with that of an equimolar solution of G-6-P. (*b*) Cysteine-carbazole method. Fructosemonophosphate formation was determined by this method (Dische & Borenfreund, 1951) on a trichloroacetic acid filtrate of the R-5-P digest. (*c*) Reduction of TPN. In earlier experiments, reduction of TPN was followed spectrophotometrically at 340 mμ. on addition of 0·5 mg. TPN, 0·4 ml. liver fraction *A* (for substrate specificity see Fig. 9) and 0·25 ml. 0·25 M-sodium phosphate buffer (pH 7·4) to 1 ml. neutralized trichloroacetic acid filtrate of the R-5-P digest in a total volume of 2·5 ml. Since liver fraction *A* is not absolutely specific for G-6-P and F-6-P, in later experiments it was replaced by purified Zwischenferment. The reaction mixture consisted of 0·5 ml. neutralized trichloroacetic acid filtrate of the R-5-P digest, 0·5 ml. 0·25 M-glycylglycine buffer (pH 7·4), 0·1 ml. 0·3 M-$MgCl_2$, 0·5 mg. TPN and 200 μg. Zwischenferment in a total volume of 2·5 ml. Under these conditions G-6-P (or F-6-P) can be estimated quantitatively over a range of 0·025–0·15 ml. 0·025 M. The reaction was started by the addition of the enzyme to both cells, the blank being devoid of TPN.

Other determinations. Pentose was determined by the orcinol method of Mejbaum (1939) with a 45 min. heating period (Albaum & Umbreit, 1947), ketopentose and ketohexose by the cysteine-carbazole method of Dische & Borenfreund (1951), glycolaldehyde by the diphenylamine method of Dische & Borenfreund (1949), glycollic acid by the chromotropic acid method of Fleury, Courtois & Perlès (1951) and phosphate by the method of Fiske & Subbarow (1925).

Preparation of ketopentose-1-phosphate. Sodium triosephosphate (from 290 mg. barium salt) was incubated at 37° for 1 hr. with 50 mg. crystalline muscle aldolase, 120 mg. glycolaldehyde and 10 ml. veronal acetate buffer (pH 7·0) in a total volume of 40 ml. After protein precipitation with $HClO_4$, the filtrate was neutralized and one-ninth of the total volume of 10 N-NaOH added. After 20 min. at room temperature (to hydrolyse any unchanged triosephosphate) it was adjusted to pH 8·0 and water-insoluble barium salts were precipitated by the addition of 4 ml. 20% (w/v) barium acetate solution. These were discarded and the barium pentosephosphate precipitated from solution by the addition of ethanol to give a final concentration of 80% (v/v). After keeping it at 0° for 2 hr., the precipitate was centrifuged, washed with ethanol and acetone and finally dried. Yield: 168 mg. barium salt. It was purified by reprecipitating with ethanol from a small volume of water. (Found (after drying *in vacuo* at 110°): organic P, 7·35; inorganic P, 0·0%.) It was completely water-soluble and contained no detectable aldose (hypoiodite method of Robison & King, 1931). Ketopentose was detected by the cysteine-carbazole and orcinol reactions (see Fig. 5). The reducing activity determined according to Nelson (1944) was the same as that of R-5-P, whereas the pentose estimated colorimetrically was only about 50% of the expected value. This behaviour in the orcinol reaction is probably general for ketopentoses since Horecker, Smyrniotis & Seegmiller (1951) found that the extinction coefficient of ribulose at 670 mμ. was only 50% of that given by aldopentoses. The constitution is discussed later.

RESULTS

Oxidation and breakdown of ribose-5-phosphate by liver fractions

The ability of the liver fractions, used in most of the experiments in the present study, to oxidize G-6-P, 6-PG, R-5-P and *iso*citrate is shown in Table 1, the TPN specificity of the oxidation of R-5-P having already been established (Dickens & Glock, 1951). These results corroborate the previous findings that at least two factors are necessary for the oxidation of R-5-P, although in this case fractions *O*, *A* and *B* singly produce some oxidation of this substrate. By further fractional precipitation with ammonium sulphate it is possible to obtain fractions which singly are completely inactive and which have to be combined to produce any oxidation (Dickens & Glock, 1951). The fractions most active in oxidizing R-5-P are clearly those with the highest G-6-P dehydrogenase activity.

Table 1. *The oxidation of* D-*glucose-6-phosphate, 6-phosphogluconate,* D-*ribose-5-phosphate and* D-*isocitrate by horse-liver fractions*

(Total volume 3·0 ml. including 1 ml. (or 0·5 ml.*) liver fraction, 0·2 ml. 0·05 M substrate and 0·2 mg. TPN. The values have been corrected for the O_2 uptake in the absence of TPN which in all cases was very small.)

Liver fraction	O_2 uptake (μl. O_2 in 45 min. at 37°)			
	G-6-P	6-PG	R-5-P	*iso*Citrate
O	42	1	11	—
A	122	22	40	132
B	10	64	10	75
C	0	89	0	1
A + *B*	—	—	146	—
A + *C*	—	—	133	—
*A** + *B** + *C**	—	—	90	—
O + *C*	—	—	97	—
B + *C*	—	—	19	—

The breakdown of R-5-P, as determined by pentose disappearance, by the same liver fractions is shown in Fig. 1. Fraction *B* is the most active and fraction *C* the least active in this respect. The most vigorous oxidation of R-5-P is, as one would expect, produced by fraction *A* which is highest in G-6-P dehydrogenase in combination with fraction *B*, the most active in splitting R-5-P. Although the oxidation of R-5-P is completely TPN specific, the breakdown is independent of the presence of coenzyme. The breakdown is, moreover, the same under aerobic and anaerobic conditions and never proceeds beyond 66–72% disappearance of pentose. This indicates the existence of some equilibrium

which is unaffected either by increasing the concentration or the duration of action of the enzyme. Crystalline muscle aldolase is completely inactive in splitting R-5-P.

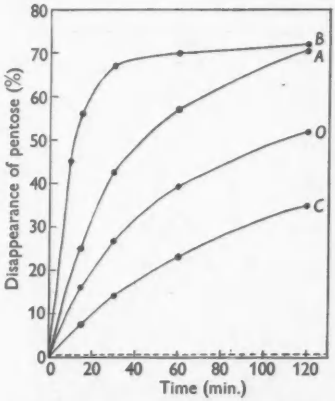

Fig. 1. Breakdown of ribose-5-phosphate by liver fractions and crystalline muscle aldolase at 37°. Digestion mixture (see Methods section) included 0·25 ml. 0·05 M-R-5-P, 1 ml. horse-liver fractions *O*, *A*, *B* or *C* or 10 mg. crystalline muscle aldolase, in total volume of 2·5 ml. Pentose disappearance measured by orcinol method. ——, horse-liver fractions; - - -, crystalline muscle aldolase.

Direct oxidation of ribose-5-phosphate to 5-phosphoribonic acid

Since direct oxidation of R-5-P to 5-phosphoribonic acid followed by oxidative decarboxylation has been suggested by Dickens (1936, 1938) to occur in yeast, the ability of liver fractions to oxidize 5-phosphoribonic acid has been investigated. It has been found, however, that combined ($A+B+C$) liver fractions do not oxidize 5-phosphoribonic acid at all, even in the presence of TPN, although such liver fractions always oxidize R-5-P. It is thus unlikely that this direct oxidative pathway exists in liver.

Formation of ketopentosephosphate by liver fractions

Ketopentose formation has been detected both from the oxidative decarboxylation of 6-PG and during the initial stages of R-5-P breakdown by liver fractions. In this connexion, the recently published cysteine-carbazole method of Dische & Borenfreund (1951) has proved particularly useful. Ketopentoses give a pink colour with maximum absorption at 545 mμ. in this reaction and also show absorption at 540 mμ. in the orcinol reaction in addition to the much greater absorption at 670 mμ. characteristic of pentoses in general. When R-5-P was incubated with liver fraction *B* at 37°, no ketopentose was detected even during the first few minutes. If, however, incubation was carried out at 28° in order to slow down the breakdown of R-5-P, which has a very high temperature coefficient (Dickens & Glock, 1951), ketopentose was detected.

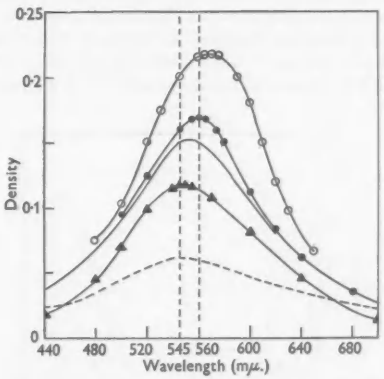

Fig. 2. Ketopentosephosphate formation from ribose-5-phosphate at 28° detected by cysteine-carbazole reaction. Digestion mixture (see Methods section) included 0·25 ml. 0·05 M-R-5-P and 1 ml. horse-liver fraction *B* in total volume of 2·5 ml. Incubation period: - - -, 0 min.; ▲—▲, 5 min.; ——, 10 min.; ●—●, 30 min.; ○—○, 120 min. (545 mμ. peak = ketopentose. 560 mμ. peak = ketohexose).

Fig. 2 gives absorption curves obtained with the cysteine-carbazole reaction after 5, 10, 30 and 120 min. incubation periods, the percentage breakdown of R-5-P at these times being, respectively, 21, 39, 60 and 71. The presence of the 545 mμ. ketopentose maximum is quite evident after 5 and 10 min., but after 30 min. the maximum has shifted to 560 mμ., characteristic of ketohexose. This ketopentose does not appear to be alkali-labile since the 545 mμ. maximum is still shown after treatment with N-sodium hydroxide for 10 min. at room temperature, although under these conditions ribulose-5-phosphate no longer gives a ketopentose reaction. Identification of the ketopentosephosphate, however, awaits its isolation.

Ketopentose is also formed from the oxidative decarboxylation of 6-PG. After measuring the rate of oxidation of 6-PG with liver fraction *C* at 37° for 60 min., pentose was determined, after trichloroacetic acid precipitation. In this instance 'apparent' pentose formation accounted for 28% of the oxidized substrate, but since ketopentose was present, which gives a much lower extinction coefficient at 670 mμ. in the orcinol reaction than aldopentose, the 'true' pentose formation must be greater than this. Fig. 3 shows the absorption curves of the digestion mixtures before and after incubation, the 670 mμ.

peak in the unincubated mixture being due to TPN. Fig. 3 also includes absorption curves of R-5-P and an equilibrium mixture of ribulose-5-phosphate and R-5-P. Both ribulose-5-phosphate and the incubated digest give an absorption maximum at 540 mμ., characteristic of ketopentoses. The rate of colour development of the incubated digest in the orcinol reaction is given in Table 2, and is very similar to that of R-5-P, which Horecker *et al.* (1951) state is identical with that of ribulose-5-phosphate.

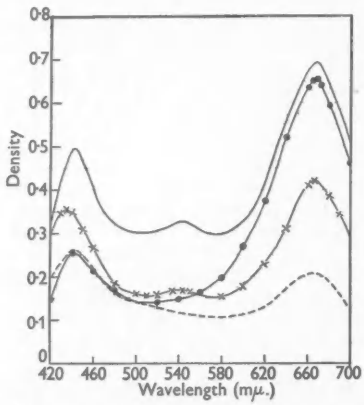

Fig. 3. Ketopentosephosphate formation from 6-phosphogluconate at 37° detected by orcinol method. Digestion mixture included 0·2 ml. 0·05 M-6-phosphogluconate and 1 ml. horse-liver fraction *C* in total volume of 2·5 ml. - - -, unincubated digestion mixture; ——, after 60 min. incubation; ●—●, 100 μg. R-5-P (barium); ×—×, 50 μg. equilibrium mixture of ribulose-5-phosphate and R-5-P (barium salts). (540 mμ. peak = ketopentose.)

Aldolase condensations with glycolaldehyde

Since R-5-P breakdown products are very readily converted into hexosemonophosphate without any accumulation of ketopentosephosphate, it was decided to attempt to synthesize ketopentosephosphate from liver fraction *B*, triosephosphate and glycolaldehyde. Ketopentosephosphate formation was very rapid and almost quantitative, the absorption curves in the orcinol and cysteine-carbazole reactions being given in Fig. 4. No hexosemonophosphate was formed. This ketopentosephosphate synthesized with liver fraction *B* is

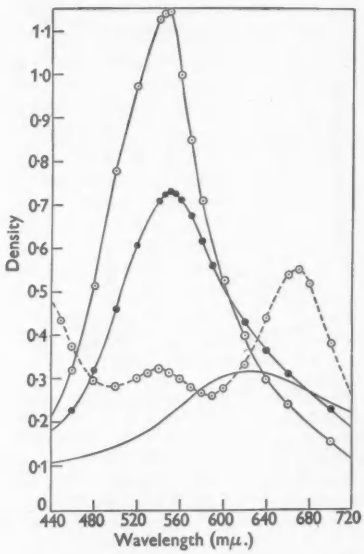

Fig. 4. Ketopentose-1-phosphate formation from triosephosphate, glycolaldehyde and horse-liver fraction *B* at 37°. Digestion mixture included 1·5 ml. 0·025 M-triosephosphate and glycolaldehyde and 0·5 ml. liver fraction *B* in total volume of 2·5 ml. Cysteine-carbazole reaction: ——, unincubated digestion mixture; ●—●, after 30 min. incubation; ⊙—⊙, after 120 min. incubation. ⊙- - -⊙, after 120 min. incubation by orcinol method.

probably the same as that synthesized under the same conditions with crystalline muscle aldolase (see Fig. 5 for absorption curves and Methods section for method of preparation and analysis). Both are alkali-stable under the conditions which

Table 2. *Rate of colour development in the pentose orcinol reaction*

	Time of boiling (min.)							
	5	10	15	20	30	45	60	90
	Colour development (% of maximum)							
Ribose-5-phosphate	37	64	78	96	100	—	—	—
Arabinose-5-phosphate	—	24	—	47	62	81	92	100
Ketopentose-1-phosphate*	33	67	82	94	100	—	—	—
6-Phosphogluconate digestion mixture†	34	68	—	89	94	100	—	—

* Synthesized from triosephosphate and glycolaldehyde with crystalline muscle aldolase.

† After determining O_2 uptake at 37° for 1 hr. in a system including 1 ml. liver fraction *C*, 0·2 ml. 0·05 M-6-PG and 0·2 mg. TPN, pentose determined in neutralized trichloroacetic acid filtrate.

destroy ribulose-5-phosphate. The rapid rate of acid hydrolysis (Fig. 6) indicates that it is a ketopentose-1-phosphate and is presumably the same as that synthesized by Racker (1948), which Dische &

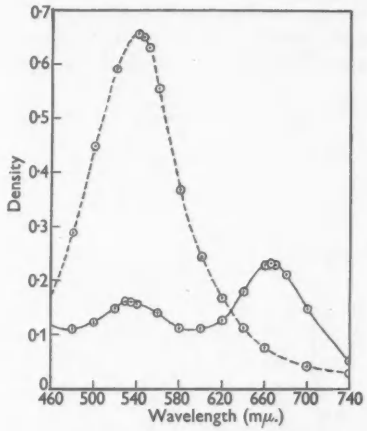

Fig. 5. Ketopentose-1-phosphate synthesized from triosephosphate and glycolaldehyde with crystalline muscle aldolase. For preparation see Methods section. 100 μg. barium salt: ⊙- -⊙, cysteine-carbazole reaction; ⊙—⊙, orcinol reaction.

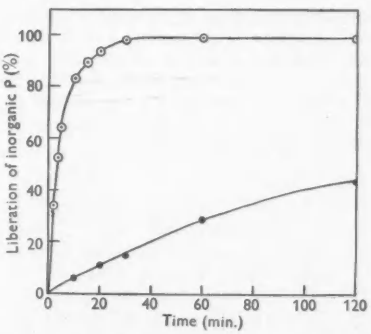

Fig. 6. Rates of hydrolysis of pentosephosphates in N-HCl at 100°. ⊙—⊙, ketopentose-1-phosphate synthesized from glycolaldehyde and triosephosphate with crystalline muscle aldolase ($k = 86 \times 10^{-3}$). ●—●, ribose-5-phosphate (synthetic) ($k = 2{\cdot}5 \times 10^{-3}$).

Borenfreund (1951) refer to as xyloketose-1-phosphate. Racker (1948), however, gave no analysis or characterization of the sugar, but Forrest, Hough & Jones (1951) have synthesized it with pure aldolase and identified the sugar chromatographically as xyloketose.

This ketopentose-1-phosphate is oxidized slowly by liver fractions. Fig. 7 shows the oxidation of R-5-P and ketopentose-1-phosphate by combined liver fractions *A* and *C*. Whereas the oxidation of R-5-P is negligible in the absence of added TPN, the slow oxidation of ketopentose-1-phosphate in the absence of TPN accounts for about half of the total oxygen uptake in the presence of TPN. The initial breakdown of ketopentose-1-phosphate presumably leads to formation of glycolaldehyde and triosephosphate, which could be synthesized to F-6-P by the action of aldolase. The oxidation of the glycolaldehyde is independent of TPN (see later) whilst the oxidation of the latter is TPN-specific.

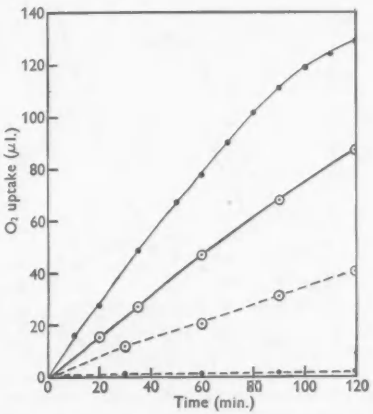

Fig. 7. Oxidation of ketopentose-1-phosphate and ribose-5-phosphate by liver fractions. O_2 uptake determined at 37°. Total volume 3·0 ml. including 1 ml. each of liver fractions *A* and *C*, 0·1 ml. 0·05 M-substrate with and without 0·4 mg. TPN. ——, with TPN; - - -, without TPN; ●, R-5-P; ⊙, ketopentose-1-phosphate.

Since it seemed theoretically possible that pentosephosphate might condense with glycolaldehyde to form a heptosephosphate, liver fraction *B* was incubated with R-5-P and glycolaldehyde, but with essentially the same results as with triosephosphate and glycolaldehyde. Again, an alkali-stable ketopentosephosphate was formed and no hexosemonophosphate or ketoheptosephosphate. As Fig. 8 shows, mannoketoheptose gives an absorption maximum in the cysteine-carbazole reaction at 565 mμ. and in the orcinol reaction at 570 mμ. in the presence of ferric chloride and a much smaller one at 595 mμ. in the absence of ferric chloride. If, however, the orcinol reaction product is extracted into amyl alcohol, the maximal absorption both in the presence and absence of iron is at 660 mμ., which is in approximate agreement with the result of Robison, Macfarlane & Tazelaar (1938) who used a similar procedure. These negative results, however, do not exclude the possibility of

the formation of heptosephosphate or ketoheptosephosphate from the breakdown products of R-5-P. Although fructose and fructose-6-phosphate give an absorption maximum at 560 mμ. in the cysteine-carbazole reaction, the maximum absorption with the R-5-P digestion mixtures is often at 565–570 mμ. (see the curve after 120 min. in Fig. 2), which would correspond more closely with a mixture of ketohexose and ketoheptose.

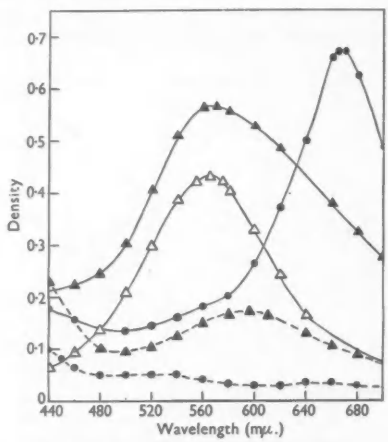

Fig. 8. Cysteine-carbazole and orcinol reactions with mannoketoheptose and ribose. Orcinol reaction with 40 μg. ribose (●) and 100 μg. mannoketoheptose (▲); ——, in presence of $FeCl_3$ in a final concentration of 0·048%; - - -, in absence of $FeCl_3$; △, cysteine-carbazole reaction with 100 μg. mannoketoheptose (ribose does not react).

Breakdown products of ribose-5-phosphate

It is generally considered that R-5-P is split enzymically into triosephosphate and a C_2 fragment, as yet unidentified, but assumed to be glycolaldehyde.

Experiments devised to detect the formation of glycolaldehyde from R-5-P liver fractions have in all cases been negative, whether carried out under aerobic or anaerobic conditions, although the Dische (1949) diphenylamine reaction is extremely sensitive. This is not due to rapid oxidation to glycollic acid. Moreover, glycolaldehyde does not disappear when incubated with fraction *B*. As already described, when glycolaldehyde is incubated with liver fraction *B* and R-5-P or triosephosphate, a ketopentose-1-phosphate accumulates very rapidly. This does not accumulate when R-5-P is split by fraction *B* and, moreover, the addition of glycolaldehyde completely inhibits the resynthesis of hexosemonophosphate from the R-5-P breakdown products. This would indicate that free glycolaldehyde is not formed during R-5-P breakdown, although the rapid synthesis of ketopentosephosphate is further evidence that the C_3 fragment is triosephosphate.

Additional evidence for triosephosphate formation is provided by the fact that DPN is reduced in addition to TPN in the presence of R-5-P and liver

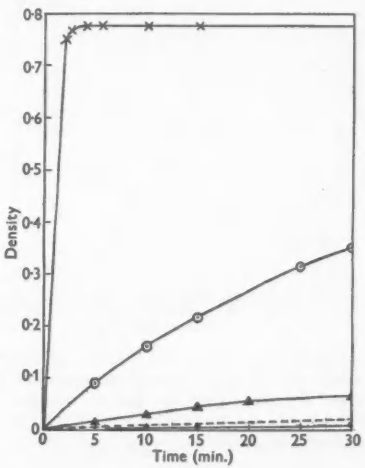

Fig. 9. Substrate specificity of liver fraction *A* used for hexosemonophosphate assay. Reduction of TPN measured at 340 mμ. at 20° in system containing 0·1 ml. 0·05 M substrate, 0·5 mg. TPN and 0·4 ml. liver fraction *A* in total volume of 2·5 ml. ×—×, D-*iso*citrate; ⊙—⊙, G-6-P or F-6-P; ▲—▲, 6-phosphogluconate; - - -, glycolaldehyde; ●—●, R-5-P.

fractions. Sable (1951) has shown that DPN reduction can be inhibited by poisoning the triosephosphate dehydrogenase with iodoacetate, whereas the TPN specific oxidation is unaffected (Sable, 1951; Dickens & Glock, 1951).

Glycolaldehyde is oxidized slowly by combined $(A+B+C)$ liver fractions, but this oxidation (which in three experiments averaged 70 μl. oxygen in 90 min. from 0·2 ml. 0·05 M substrate) is unaffected by the addition of DPN or TPN. Breusch (1943) has also reported oxidation of glycolaldehyde by liver suspensions.

Fig. 9 shows the rate of reduction of TPN on the addition of D-*iso*citrate, G-6-P, 6-PG and a R-5-P digestion mixture to a liver fraction *A*, but no reduction is produced on the addition of either glycolaldehyde or R-5-P. This, and the previous evidence, proves conclusively that the TPN-specific oxidation of the breakdown products of R-5-P is not due, even in part, to the oxidation of free glycolaldehyde.

Table 3. *Hexosemonophosphate formation from ribose-5-phosphate by horse-liver fraction B*

(For composition of R-5-P digestion mixture and for details of hexosemonophosphate determination see Methods section: (1) by O_2 uptake with liver fraction *A*; (2) cysteine-carbazole method; (3) reduction of TPN at 340 mμ. with liver fraction *A*; (4) reduction of TPN at 340 mμ. with purified Zwischenferment.)

Exp. no.	Time of incubation at 37° (min.)	Pentose disappearance (%)	Percentage conversion of R-5-P broken down into	
			Hexosemono-phosphate	Fructosemono-phosphate (2)
1	60	70	80 (1) 74 (3)	—
2	60	38*	83 (1) 78 (3)	—
3	5†	22	—	64
	15	40	—	45
	120	71	—	30
4	5	40	—	68
	10	60	—	44
	30	66	—	29
5	5	35	—	68
	15	58	—	45
	60	72	—	29
6	30	69	73 (4)	28
	60	69	78 (4)	30
	120	68	75 (4)	28

* A less active enzyme. † Incubated at 28°.

Synthesis of hexosemonophosphate

Synthesis of hexosemonophosphate from the products of R-5-P breakdown by liver fraction *B* has been determined by measuring the oxygen uptake on addition of TPN and liver fraction *A* (rich in G-6-P dehydrogenase but completely inactive in oxidizing R-5-P, see Fig. 9), or by measuring the reduction of TPN at 340 mμ. in the presence of liver fraction *A* or purified Zwischenferment. Both these enzyme preparations oxidized F-6-P and G-6-P at identical rates so that the above methods determine total hexosemonophosphate. Fructosemonophosphate was determined in addition, in some cases, by the cysteine-carbazole reaction. Table 3 gives the results of these experiments. The average conversion of the R-5-P broken down into hexosemonophosphate is at least 75 %, which is much greater than the 50 % yield obtainable by simple aldolase condensation of triosephosphate derived from R-5-P. The results obtained with the cysteine-carbazole method indicate that fructosemonophosphate is formed first and is gradually converted into G-6-P, an equilibrium mixture of these two esters being formed after about 30 min. incubation, as is seen by the proportion of fructosemonophosphate to total hexosemonophosphate (Table 3).

DISCUSSION

From the results of the present and previous work (Dickens & Glock, 1951) it would appear that at least three enzymes are necessary for the oxidation of ribose-5-phosphate (R-5-P) by liver fractions. Since R-5-P is not split by crystalline muscle aldolase, and ketopentosephosphate formation precedes both formation and breakdown of R-5-P by liver fractions, both an aldolase and a pentosephosphate isomerase are required for the breakdown of R-5-P. Hexosemonophosphate dehydrogenase is necessary, in addition, for the oxidation of R-5-P. Horecker & Smyrniotis (1950) and Horecker *et al.* (1951) have indicated the occurrence of a pentosephosphate isomerase, concerned with the interconversion of ribulose-5-phosphate and R-5-P, in phosphogluconic acid dehydrogenase preparations from yeast. Their statement that the ribulose is in the form of the 5-phosphate is not based on evidence which would exclude the 1-phosphate, and since liver contains a phosphoribomutase (Abrams & Klenow, 1951*a*, *b*) which converts ribose-1-phosphate into R-5-P, it is conceivable that it might also contain a pentosephosphate isomerase for converting ribose-1-phosphate into ribulose-1-phosphate. In addition, another enzymic equilibrium must exist since R-5-P is never broken down by liver fractions beyond 66–72 % disappearance of pentose.

Approximately 75 % of the whole carbon skeleton of R-5-P broken down by liver fraction *B* has been accounted for by conversion into hexosemonophosphate. Fructosemonophosphate is formed first, and is gradually converted into G-6-P. Synthesis of fructosemonophosphate cannot proceed via fructosediphosphate since, as previously shown (Dickens & Glock, 1951), liver fractions which readily oxidize

both F-6-P and G-6-P do not oxidize fructosediphosphate, these fractions all being deficient in fructosediphosphatase. Fructosediphosphate, if formed at all, can only be present as a minor component. Dische (1949) also showed that hexosemonophosphate was formed from R-5-P by haemolysed erythrocytes independently of fructosediphosphate formation. The high yield of hexosemonophosphate indicates that it cannot only be formed by aldolase condensation of two triose molecules derived from two molecules of R-5-P and at present one can only speculate as to how it might occur. Dische (1951) postulates not only the formation of tetrose and tetrosephosphate but also of C_7 sugar phosphates. In spite of the fact that no free glycolaldehyde has been detected during the breakdown of R-5-P by liver fractions, it is conceivable that it might condense immediately it is formed with a molecule of glycolaldehyde phosphate to give a tetrosephosphate, and this possibility is being investigated. It does not seem possible that two glycolaldehyde molecules would condense since glycolaldehyde does not disappear when incubated with liver fraction *B*, the most active in splitting R-5-P. Hough & Jones (1951*a–c*) have emphasized the possible importance of glycolaldehyde as a sugar precursor, but all their experiments were carried out under mildly alkaline conditions in the absence of enzymes. The only enzymic condensation of glycolaldehyde so far reported is with triosephosphate (Racker, 1948; Forrest *et al.* 1951; and the present paper) and irrespective of the source of aldolase the product is xyloketose-1-phosphate, which has not yet been shown to occur naturally. Marmur & Schlenk (1951) found that both glycolaldehyde and glycolaldehydephosphate condensed with triosephosphate in the presence of an enzyme preparation from *Micrococcus pyogenes*, but the pentosephosphate was not characterized.

The possibility suggested by Fig. 2 that heptosephosphate might be synthesized in addition to hexosemonophosphate is also of interest, particularly since Robison *et al.* (1938) isolated a ketoheptosephosphate from the products of yeast-juice fermentation and more recently Benson, Bassham & Calvin (1951) have found that sedoheptulosemonophosphate is formed during photosynthesis and is indeed a normal constituent of all plants.

SUMMARY

1. The breakdown of ribose-5-phosphate (R-5-P) by dialysed ammonium sulphate-precipitated horse-liver fractions is independent of coenzyme II (TPN), the same under aerobic and anaerobic conditions and never proceeds beyond 66–72% pentose disappearance.

2. Oxidation of R-5-P is completely TPN-specific and is greatest with a combination of liver fraction *B*, most active in splitting R-5-P, and fraction *A*, which has the highest glucose-6-phosphate dehydrogenase activity.

3. Crystalline muscle aldolase does not split R-5-P.

4. Ketopentosephosphate formation has been detected both from the oxidative decarboxylation of 6-phosphogluconate and during the initial stages of breakdown of R-5-P by liver fractions.

5. Attempts to synthesize ketopentosephosphate from triosephosphate and glycolaldehyde with liver fractions have resulted in the rapid accumulation of a ketopentose-1-phosphate probably the same as that synthesized by crystalline muscle aldolase under the same conditions. This does not accumulate during the breakdown of R-5-P by liver fractions.

6. The main product of R-5-P breakdown by liver fractions is hexosemonophosphate which accounts for approximately 75% of the pentose disappearing. This conversion is much greater than could be accounted for by aldolase condensation of triosephosphate derived from R-5-P, and indicates that the rest of the pentose molecule must be incorporated.

7. Fructosemonophosphate is formed first and is then gradually converted into glucose-6-phosphate. Synthesis of fructosemonophosphate does not proceed via fructosediphosphate.

8. Free glycolaldehyde is not formed during the breakdown of R-5-P by liver fractions and the TPN-specific oxidation of the breakdown products cannot be due, even in part, to oxidation of glycolaldehyde.

9. Direct oxidation of R-5-P to 5-phosphoribonic acid followed by oxidative decarboxylation is unlikely to occur in liver since 5-phosphoribonic acid is not oxidized by liver fractions even in the presence of TPN.

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Comparative Studies of 'Bile Salts'

5. BILE SALTS OF CROCODYLIDAE

BY G. A. D. HASLEWOOD
Guy's Hospital Medical School, S.E. 1

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The Crocodylidae are reptiles which are believed to have persisted without much alteration for a very long period. Accordingly it was expected, on the hypotheses put forward in these studies, that their bile salts would show primitive characteristics. A brief report of work on *Alligator mississippiensis* has already appeared (Haslewood, 1952*a*) and details of this, together with some findings in two other species, namely the spectacled cayman (*Caiman crocodilus*) and the Nile crocodile (*Crocodylus niloticus*) are now presented.

RESULTS

The work described shows that the bile acids of each of the three species examined are present in similar complex mixtures. Crystalline material was easily obtained, but this, too, was very impure. By chromatography on alumina it proved in every case simple to isolate the ethyl ester of an acid $C_{27}H_{46}O_5$, named 'trihydroxycoprostanic acid'; this was further characterized as the parent acid and its methyl ester. The dehydro derivatives obtained after oxidation with chromic acid contained three carbonyl groups in the molecule. These groups could be removed by Kishner-Wolff reduction, which gave the 'stem acid', coprostanic acid, $C_{27}H_{46}O_2$; and this has been prepared by partial synthesis from cholanic acid (Bridgwater & Haslewood, 1952).

Through the kindness of Prof. T. Shimizu of the University of Okayama Medical School, Japan, it has been possible to show that trihydroxycoprostanic acid is identical with the 'α-trihydroxybisnorsterocholanic acid', isolated by Kurauti & Kazuno

(1939) from the bile of the frog *Rana catesbiana*. This finding suggests a change in nomenclature, which is discussed below.

EXPERIMENTAL

General. All melting points are uncorrected. Al_2O_3 was obtained from Hopkin and Williams and neutralized (Shoppee, 1949). Micro-analysis was done by Weiler and Strauss, Oxford. Optical rotations were determined in a 1 dm. microtube. 20% CrO_3 is a solution made by dissolving CrO_3 (20 g.) in the minimal amount of water and making up to 100 ml. with acetic acid. H-test = Hammersten's HCl test (Haslewood, 1943). Light petroleum, 'b.p. 40–60°'.

Fractionation of bile acids

The methods described by Haslewood & Wootton (1950) were followed. In all species, liquors from the hydrolysis of the bile salts were tested with $BaCl_2$ for SO_4^{2-}. Only small amounts of this ion were detected.

Alligator mississippiensis. Seven gall bladders, from animals 4–8 ft. long, yielded about 16·7 g. of light-brown hygroscopic bile salts. These proved somewhat resistant to hydrolysis, and after experiments the following method was adopted: a solution of the bile salts (1 g.) in water (5 ml.) with NaOH (5 ml. of 5N) was sealed in a small metal bomb and heated at 118–120° for 16 hr. Extraction of the contents of the cooled bomb with water gave a clear brown solution which was treated with excess of dilute HCl and of solid NaCl. After some hours, the precipitated gummy bile acids were collected and washed. Part of the precipitated bile acids tended to dissolve on washing, and to be re-precipitated with the NaCl-containing liquors. This material did not appear to be unchanged bile salts, and was similar to the ethyl acetate-insoluble precipitate (below). The main washed bile acid precipitate was dissolved in ethanol and the solution evaporated. The residue, with ethyl acetate, gave an insoluble gummy solid which was filtered off and kept. This material was not, apparently, altered on further heating with alkali as above. The ethyl acetate filtrate was evaporated to small bulk and set aside for crystallization.

The crystals were collected after 4–5 days at 0–5°, washed with cold ethyl acetate and dried. The yield from six such experiments was 0·14–0·19 g. (average 0·17 g.). This material (1·5 g.) was recrystallized once from ethyl acetate and gave short white needles (0·8 g.), m.p. 160–165°. Ethylation with diazoethane gave esters (0·59 g.) and unesterified acids which were extracted from ethereal solution with dilute ammonia. The esters (0·59 g.) left on evaporation of the washed ether were dissolved in benzene and separated on Al_2O_3 (6 g.) in a column. Benzene (1245 ml.) eluted a total of 0·35 g. which crystallized on standing with light petroleum. Ether (105 ml.) then eluted 0·15 g. and ethanol (40 ml.) 0·07 g. from the column. Total eluted, 0·57 g. The 0·35 g. of material eluted with benzene was *ethyl trihydroxycoprostanate*; the purest samples obtained had m.p. 143·5–144·5°, and crystallized from light petroleum/benzene in small colourless leaflets or flat needles. $[\alpha]_D^{21°} + 28 \pm 2°$ in ethanol (*c*, 1·7). (Found: C, 72·4; H, 10·2. $C_{29}H_{50}O_5$ requires C, 72·8; H, 10·5%.) This substance was more easily eluted from Al_2O_3 than ethyl cholate. Further samples of this ester were obtained by re-chromatography of the material eluted with ether (elution with benzene removed the ethyl trihydroxycoprostanate) and also, by the same procedure, after re-esterification of the acid not esterified by the first treatment with diazoethane.

In general, elution of the ethyl esters from Al_2O_3 with benzene removed most of the ethyl trihydroxycoprostanate, and with ether and then ethanol material was obtained which was a mixture. Such material was recrystallized from ethanol/water to give long white needles, m.p. 200–203°, which were still not sufficiently pure to give satisfactory analytical figures. (Found: C, 71·5; H, 10·6%.)

The ethyl acetate liquors from which the crystalline acids had been filtered were evaporated and the gummy residue (4·35 g.) was twice treated with diazoethane and separated into esters and unesterified acids as described above. The combined ester fraction (3·0 g.) was dried over H_2SO_4 *in vacuo*; it was then dissolved in ethanol (30 ml.) with Girard's reagent 'T' (1·0 g.) and acetic acid (3 ml.). The mixture was boiled under reflux for 80 min.; then partially neutralized and separated into ketones (0·062 g.) and non-ketones (2·85 g.) in the usual way. The ketonic fraction, on separation on Al_2O_3 (1 g.) in a column, gave a small amount of crystalline material. The non-ketones, treated similarly, gave no easily identifiable crystalline material.

Caiman crocodilus. One gall bladder gave about 2·5 g. hygroscopic bile salts which were treated exactly as those of the alligator. The crude ethyl acetate crystals from the bile salts (2 g.) weighed 0·36 g., and had m.p. 169–172°. These were dissolved in ethanol (7 ml.) with H_2SO_4 (0·1 ml.) and the solution left at approx. 22° for 16 hr. Dilution with $NaHCO_3$ solution precipitated the crude ethyl esters (0·34 g.), which were filtered off. (The filtrate on acidification gave a little unesterified acid, which crystallized on standing.) The esters (0·34 g.) were separated on Al_2O_3 as described above and, on elution with benzene, yielded ethyl trihydroxycoprostanate (0·19 g.), m.p. 138–140° (not depressed by mixture with the ester from alligator bile), together with (probably) more hydroxylated esters (0·14 g.) eluted with ether and ethanol. Ethyl triketocoprostanate (see below) from the above ester (m.p. 138–140°) had m.p. 203–206°; coprostanic acid (see below), m.p. 101–104°, was prepared from the triketo ester.

Crocodylus niloticus. One gall bladder gave about 0·7 g. of light-brown hygroscopic bile salts. These were treated as described above. The crude crystals (0·12 g.) from ethyl acetate had m.p. 162–168°. The ethyl trihydroxycoprostanate, prepared as above, had m.p. 138–140° (not depressed by mixture with the ester from alligator bile); the derived ethyl triketocoprostanate had m.p. 203–204° and the coprostanic acid had m.p. 100–103°.

Trihydroxycoprostanic acid and its derivatives

Substances which are probably identical with those previously described by Kurauti & Kazuno (1939) or Mabuti (1941*b*) under different names are indicated by an asterisk.

*Trihydroxycoprostanic acid**. Ethyl trihydroxycoprostanate (100 mg.), ethanol (1 ml.) and KOH solution (0·1 ml. of 40% w/v) were gently boiled together under reflux for 15 min. The solution was diluted with water and acidified with HCl. The precipitated acid was collected, washed with water and dissolved in ethanol. Evaporation of the filtered solution under N_2 in a test tube left a residue which was crystallized from ethyl acetate (1–2 ml.). *Trihydroxycoprostanic acid* formed short white needles which melted at 167–168° to a cloudy liquid which cleared

sharply at 174°. These apparently contained some solvent, or water, for it was necessary to dry to constant weight at 80° to obtain satisfactory analytical figures. (Found: C, 72·2; H, 10·5; equiv. by titration, 446. $C_{27}H_{46}O_5$ requires C, 72·0; H, 10·2%; mol.wt., 450.) Trihydroxycoprostanic acid crystallized from ether in hexagonal prisms (m.p. 171–173°) very similar to those obtainable from cholic acid. It gave a blue-green colour in the H-test.

Methyl trihydroxycoprostanate. A solution of the above acid (50 mg.) in methanol (1 ml.) with H_2SO_4 (1 drop) was allowed to stand at about 22° for 4 days. Dilution with aqueous $NaHCO_3$ gave the ester as a gum: esterification was apparently quantitative. The ester, in benzene, was eluted from Al_2O_3 (0·5 g.) with benzene and the product recrystallized from light petroleum/benzene, from which *methyl trihydroxycoprostanate* separated as small white needles, m.p. 153–155°. (Found: C, 72·5; H, 10·5. $C_{28}H_{48}O_5$ requires C, 72·4; H, 10·4%.)

Ethyl triketocoprostanate. Ethyl trihydroxycoprostanate (0·1 g.) in acetic acid (1·5 ml.) was treated with 20% CrO_3 (0·3 ml.), added gradually with efficient cooling in cold water (this was necessary to obtain a product of sharp melting point). After 10 min. at about 20°, the solution was diluted with water and the product isolated by filtration and thoroughly washed with water. It was crystallized from dilute ethanol, from which *ethyl triketocoprostanate* separated as fine colourless needles of m.p. 205–206°. (Found: C, 73·6; H, 9·3. $C_{29}H_{44}O_5$ requires C, 73·7; H, 9·3%.)

Methyl triketocoprostanate. This was similarly prepared from methyl trihydroxycoprostanate and crystallized from dilute ethanol in small colourless needles which had m.p. 222–224°. (Found: C, 72·9; H, 9·1. $C_{28}H_{42}O_5$ requires C, 73·3; H, 9·2%.)

Triketocoprostanic acid.* This was prepared by hydrolysis of ethyl triketocoprostanate and crystallized from benzene/acetone as small white needles, which had m.p. 227–228° (decomp.). (Found: C, 73·2; H, 9·1. $C_{27}H_{40}O_5$ requires C, 73·0; H, 9·0%.)

Coprostanic acid. Ethyl triketocoprostanate (34 mg.) was added to a solution, in a small metal bomb, of Na (50 mg.) in ethanol (2 ml.) with hydrazine hydrate (0·1 ml. of 90–95%). The bomb was sealed and heated at 200–207° for 4 hr. The contents of the cooled bomb were then washed out with water and acidified with HCl. The solid which separated was collected, washed with water and dissolved in methanol. The filtered solution was warmed and diluted to cloudiness with water. On standing, colourless needles (16 mg.) separated. These could be recrystallized from aqueous methanol or aqueous acetone from which *coprostanic acid* separated as long glistening colourless needles which had m.p. 103·5–105·5°; $[\alpha]_D^{21°}$ +28±2° in $CHCl_3$ (*c*, 0·7). (Found: C, 81·1; H, 11·0. $C_{27}H_{46}O_2$ requires C, 80·5; H, 11·5%.) The Kishner-Wolff reduction had to be carried out with careful attention to details of quantities, especially of Na, in order to obtain a product from which crystals could be isolated.

Comparison of trihydroxycoprostanic acid and 'α-trihydroxybisnorsterocholanic acid'

Prof. T. Shimizu kindly sent the author, at his request, 0·1 g. of the acid 'm.p. 172°' from the bile of *Rana catesbiana*. Shimizu's acid had m.p. 166–169·5°. The mixed melting point with trihydroxycoprostanic acid (m.p. 167–168°) was 163–170°. Shimizu's acid (50 mg.) was dissolved in ethan (1 ml.) with H_2SO_4 (1 drop) and the solution kept at about 21° for 2 days. Dilution with aqueous $NaHCO_3$ precipitated the ester as a semi-solid (the $NaHCO_3$ liquors gave a cloudy solution on acidification). The ester was dried by evaporation with ethanol, dissolved in benzene and absorbed on to Al_2O_3 (0·5 g.) in a slow-moving column. Benzene (40 ml.) eluted 30·7 mg., ether (25 ml.) 7·8 mg. and ethanol (25 ml.) 10·4 mg. Total eluted, 48·9 mg. The 30·7 mg. from the benzene eluate crystallized, on treatment with light petroleum, as highly purified ethyl trihydroxycoprostanate, m.p. 143·5–144·5°, not depressed by the ester (m.p. 140–142°) from alligator's bile. The ether eluate yielded a rather less pure specimen of ethyl trihydroxycoprostanate and the ethanol eluate was partially crystalline. Hence, at least about 45 mg. (90%) of Shimizu's product must have been trihydroxycoprostanic acid. Ethyl triketocoprostanate from Shimizu's trihydroxy ester had m.p. 200–202°, not depressed by material (m.p. 205–206°) prepared from alligator's bile.

DISCUSSION

Biological

The presence of a C_{27} bile acid in Crocodylidae is in accordance with the view that the family is primitive in an evolutionary sense. It is of particular interest that this acid is identical with one from the frog *Rana catesbiana*, the bile of which species contains as its chief constituent a C_{27} or C_{28} alcohol, conjugated with sulphate (Kurauti & Kazuno, 1939), as in the case of *R. temporaria* (Haslewood, 1952*b*). These findings suggest that trihydroxycoprostanic acid is not to be looked upon as a bile acid characteristic of Crocodylidae or, indeed, of any other family, but rather as an early type of bile acid which may at one time have been widely distributed in the animal kingdom, as is cholic acid amongst 'modern' vertebrates. If this is correct we may expect to find trihydroxycoprostanic acid and its relatives on many occasions in the bile of what are now regarded as 'primitive' creatures. Its presence in minor amounts in frog's bile may mean that the original alcohol-sulphate bile salt type in these animals is giving way to the now common acid-taurine type of conjugate.

Bile acids with more than twenty-four carbon atoms in the molecule have been encountered on several occasions: a list is given in Table 1. A study of this supports the view that such acids are characteristic of the bile of the more primitive vertebrates.

Chemical

Trihydroxycoprostanic acid shows an obvious general similarity to cholic acid, and this similarity even extends to one of the crystalline forms. The Hammarsten reaction is much less obvious than with cholic acid, and there is also marked resistance to ethylation. Mabuti (1941*b*) has degraded 'α-trihydroxybisnorsterocholanic acid' to cholic acid. This proves the position and configuration of the

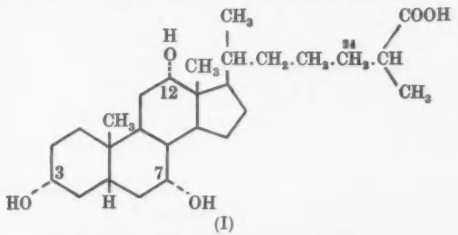
Table 1. *Bile acids with molecules containing more than twenty-four carbon atoms*

Acid	Melting point (°)	Melting point of dehydro acid (°)	Species	Reference	Remarks
$C_{27-28}H_{46-48}O_6$	255	175	A shark	Ohta (1939*a*)	—
Tetrahydroxynorsterocholanic, $C_{27}H_{46}O_6$	217	—	Teleosts:		
			Pelteobagrus nudiceps	Ohta (1939*b*)	OH at $C_{(3)}C_{(6)}C_{(12)}$ and $C_{(24)}$. Gives a positive Hammarsten reaction. Converted to *allo*cholanic acid, 12-ketocholanic acid and hyodeoxycholic acid (Ohta, 1939*b*; Isaka, 1940)
			Tetrodon porphyleus	—	
			'Bari' fish	Isaka & Azato (1940)	
			Inimicus japonicus	Mabuti (1941*a*)	
Trihydroxybufosterocholenic,* $C_{28}H_{46}O_5$	160	269	Japanese toad, *Bufo vulgaris*	Shimizu & Oda (1934)	Degraded to bisnorcholic acid (Shimizu & Kazuno, 1936)
Trihydroxy*iso*sterocholenic,* $C_{26}H_{44}O_5$	227	214	Japanese toad, *B. vulgaris*	Shimizu & Kazuno (1936)	Degraded to bisnorcholic acid (Shimizu & Kazuno, 1937)
α-Trihydroxybisnorsterocholanic,*† $C_{26}H_{44}O_5$	172	231	Frog, *Rana catesbiana*	Kurauti & Kazuno (1939), Mabuti (1941*b*)	Degraded to cholic acid (Mabuti, 1941*b*)
β-Trihydroxybisnorsterocholanic,* $C_{26}H_{44}O_5$	190–195	Identical with the above	Frog, *R. catesbiana*	Mabuti (1941*c*)	
Tetrahydroxysterocholanic, $C_{27-28}H_{46-48}O_6$	150	215	Turtle, *Amyda japonica*	Yamasaki & Yuuki (1936)	Easily forms a lactone, m.p. 208°. Degraded to bisnorcholic acid and other 3:7:12-trihydroxy steroids (Kanemitu, 1942)
			Tortoise, *Emys orbicularis*	Kim (1939)	
Tetrahydroxy*iso*sterocholanic, $C_{27-28}H_{46-48}O_6$	205	188	*Amyda japonica*	Suganami & Yamasaki (1942)	Easily forms a lactone, m.p. 220°
Trihydroxycoprostanic,† $C_{27}H_{48}O_5$	173	228	*Alligator mississippiensis*, *Caiman crocodilus*, *Crocodylus niloticus*	Present work	Carbon skeleton of coprostane; carboxyl at $C_{(26)}$ (Bridgwater & Haslewood, 1952)
Varanic, $C_{27}H_{46}O_6$	120 (hydrate)	—	Lizard, *Varanus niloticus*	Haslewood & Wootton (1950)	—

* Not the major constituents of the bile salts.

† Identical (present work).

nuclear hydroxyl groups, and, together with the work of Bridgwater & Haslewood (1952), leaves little doubt that trihydroxycoprostanic acid is 3α:7α:12α-trihydroxycoprostanic acid (I).



(I)

Note on nomenclature. Kurauti & Kazuno's name 'trihydroxybisnorsterocholanic' for the acids from *R. catesbiana* is based on a 'sterocholanic acid', $C_{28}H_{48}O_2$, of partially known structure. It is now proposed to apply a nomenclature based on coprostanic acid (a substance of known structure) to C_{27} bile acids which can be converted to this compound. Prof. T. Shimizu and Prof. T. Kazuno have generously agreed to this change in the case of 'α-trihydroxybisnorsterocholanic acid' which is therefore to be called 'trihydroxycoprostanic acid'.

SUMMARY

1. A mixture of crystalline acids was readily isolated after alkaline hydrolysis of the bile salts of three species of Crocodylidae. After ethylation of this mixture the esters could be separated on alumina. This process gave the ethyl ester of an acid, $C_{27}H_{46}O_5$, and a mixture of esters of (probably) more hydroxylated acids. The acid $C_{27}H_{46}O_5$ was prepared from the ethyl ester and has been called 'trihydroxycoprostanic acid'. The corresponding triketocoprostanic acid, $C_{27}H_{40}O_5$, and some of its derivatives were prepared. Ethyl triketocoprostanate on Kishner-Wolff reduction and hydrolysis gave the 'stem acid', coprostanic acid, $C_{27}H_{46}O_2$.

2. Trihydroxycoprostanic acid is identical with the 'α-trihydroxybisnorsterocholanic acid' isolated from the bile of *Rana catesbiana* by Kurauti & Kazuno (1939). Since this substance was degraded to cholic acid by Mabuti (1941*b*), trihydroxycoprostanic acid is almost certainly 3α:7α:12α-trihydroxycoprostanic acid. With the agreement of the Japanese workers, it is suggested that 'α-trihydroxybisnorsterocholanic acid' be referred to as 'trihydroxycoprostanic acid'.

The author thanks Prof. T. Shimizu for the 'α-trihydroxybisnorsterocholanic acid'; Dr W. H. Pearlman (Jefferson Medical College, Philadelphia) and the Sabine National Wildlife Refuge (Louisiana) for alligator bile; Dr L. C. Beadle (Makerere College, Uganda) for crocodile bile and Dr R. E. Rewell, Mr W. E. Lawrence and the Zoological Society of London for the bile of the cayman.

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Comparative Studies of 'Bile Salts'

6. PARTIAL SYNTHESIS OF COPROSTANIC ACID, A 'STEM ACID' OF PRIMITIVE BILE SALTS

By R. J. BRIDGWATER and G. A. D. HASLEWOOD
Guy's Hospital Medical School, London, S.E. 1

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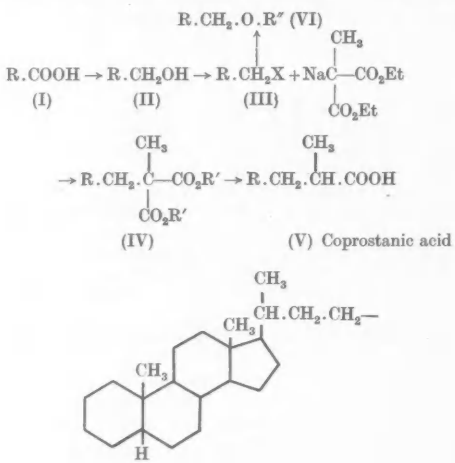
A fundamental problem in studying the bile salts of primitive animals is to provide proof of the nature of the carbon skeleton of biliary alcohols or acids whose molecules probably contain twenty-seven or twenty-eight carbon atoms. Such substances have frequently been degraded to known C_{24} bile acids or their lower homologues, but until recently no unequivocal evidence concerning a complete carbon skeleton had been published. The present authors (Bridgwater & Haslewood, 1952), in a preliminary note, reported the partial synthesis from cholanic acid of a C_{27} acid ('coprostanic acid'; coprostan-26-oic acid), also derived without loss of carbon atoms from the trihydroxycoprostanic acid isolated from the bile of Crocodylidae (Haslewood, 1952). This work is now described in detail.



R = norcholanyl

RESULTS

Cholanic acid (I) was converted by lithium aluminium hydride reduction of the ethyl ester to cholan-24-ol (II) which was converted into the corresponding chloride (III, X = Cl; Wessely & Swoboda, 1951), bromide (III, X = Br) and iodide (III, X = I). The iodide (III, X = I) was condensed with diethyl sodiomethylmalonate to give the substituted malonic ester (IV, R′ = Et) which on hydrolysis yielded the acid (IV, R′ = H). Decarboxylation of this *in vacuo* gave coprostanic acid (coprostan-26-oic acid, V), which was identical in chemical and physical properties with the stem acid, $C_{27}H_{46}O_2$, already obtained from trihydroxycoprostanic acid. The infrared spectra of the partially synthetic and 'natural' coprostanic acids were determined for us by Dr A. R. H. Cole, and curves kindly supplied by him are shown in Fig. 1. They leave no doubt about the identity of the two compounds examined. As by-products, the action of alkaline methanol or ethanol on unchanged halide (III) gave cholanyl methyl ether (VI, R″ = CH_3) and cholanyl ethyl ether (VI, R″ = C_2H_5).

EXPERIMENTAL

Melting points are uncorrected. Micro-analyses were done by Weiler and Strauss, Oxford. Optical rotations were measured in a 1 dm. steel microtube. Al_2O_3 was 'Type H', supplied by Peter Spence and Sons, Widnes. Pentane, b.p. 34–35°, was purified by repeated shaking with H_2SO_4, followed by washing with water and aqueous KOH: the material was then dried over KOH and distilled over fresh KOH.

Cholan-24-ol (II) (compare Wessely & Swoboda, 1951). To a solution of lithium aluminium hydride (7·5 g.) in dry ether (200 ml.) was added during 10 min. a solution of ethyl cholanate (17·5 g.) in dry ether (200 ml.) whilst the reaction flask was cooled with ice water. After 1 hr. at 20° the reaction mixture was slowly poured on to a mixture of crushed ice and 2 N-H_2SO_4 (excess). The ether layer was then isolated and washed with aqueous H_2SO_4, water, aqueous Na_2CO_3 (10%, w/v) and water. The ethereal extract was dried (Na_2SO_4) and evaporated to yield cholan-24-ol (9·0 g.), which on recrystallization from acetone gave needles of m.p. 128–129·5°; $[\alpha]_D^{21°} +21 \pm 2°$ in $CHCl_3$ (*c*, 3·7). (Found: C, 83·0; H, 12·1. Calc. for $C_{24}H_{42}O$: C, 83·2; H, 12·2%.) A further crop of cholan-24-ol (1·5 g.), m.p. 125–126°, was obtained from the mother liquors. A mixture of cholan-24-ol (75 mg.) in acetic acid (0·75 ml.), acetic anhydride (0·25 ml.) and 6 N-$HClO_4$ (0·3 ml.) was kept at about 20° for 0·5 hr. The solution was poured into water and the precipitated solid was crystallized from acetone, from which 24-*acetoxycholane* formed colourless needles of m.p. 85·5–86·5°. (Found: C, 80·3; H, 11·0. $C_{26}H_{44}O_2$ requires C, 80·4; H, 11·4%.)

24-Chlorocholane (III, X = Cl). Cholan-24-ol (7·5 g.) was ground with PCl_5 (5 g.) and, after 1 hr., the resulting mass was treated with water and extracted with ether. After filtration of a certain amount of ether-insoluble material, evaporation of the ether gave a brown oil which was dissolved in pentane. The solution was passed through a short column of Al_2O_3 (approx. 25 g.). Evaporation of the combined pentane eluates (about 400 ml.) yielded a white crystalline compound, recrystallization of which from acetone gave colourless prisms of 24-chlorocholane, m.p. 74–75°. (Found: C, 78·9; H, 10·7; Cl, 10·3. Calc. for $C_{24}H_{41}Cl$: C, 79·0; H, 11·3; Cl, 9·7%.)

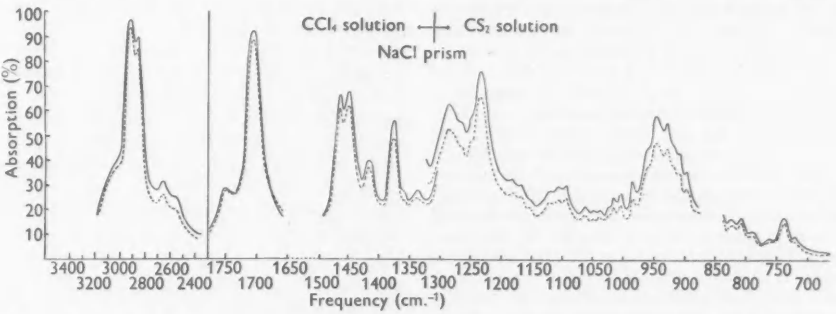


Fig. 1. Infrared spectra of partially synthetic coprostanic acid, ——; and of alligator stem acid, $C_{27}H_{46}O_2$, - - -. For experimental details see text.

24-Bromocholane (III, X = Br). A procedure identical with the above, with cholan-24-ol (523 mg.) and PBr_5 (approx. 1 g.) yielded, after chromatography, crystals (397 mg.) which on recrystallization from acetone gave *24-bromocholane* (324 mg.) as long plates, m.p. 86–86·5°. (Found: Br, 19·7. $C_{24}H_{41}Br$ requires Br, 19·5%.)

24-Iodocholane (III, X = I). Cholan-24-ol (830 mg.) was refluxed for 2 hr. with red P (90 mg.) and I_2 (1·14 g.) in toluene (15 ml.). The reaction mixture was treated with water and ether. The ether layer was washed with water, aqueous $Na_2S_2O_3$, water, aqueous $NaHCO_3$ and water, dried (Na_2SO_4) and evaporated to give a yellow crystalline solid, which was purified by chromatography in pentane on Al_2O_3. This yielded white crystals (953 mg.; 87%), recrystallization of which from acetone gave white needles of *24-iodocholane* (705 mg.), m.p. 101–102°; $[\alpha]_D^{22°}$ +23±2° in $CHCl_3$ (*c*, 2·8). (Found: I, 27·7. $C_{24}H_{41}I$ requires I, 27·8%.) A further crop (128 mg., m.p. 100·5–101·5°) was obtained from the mother liquors.

In a larger scale preparation, cholanic acid (10 g.) was esterified by slow dissolution in ethanol (200 ml.) containing H_2SO_4 (2%, v/v) and leaving at 20° for 5 hr. The reaction mixture was poured into water and the precipitated ethyl cholanate filtered off, washed and dried. This crude ester was reduced directly with lithium aluminium hydride as described and the resulting crude alcohol converted into the iodide, which was then purified by chromatography to give 24-iodocholane (10 g.). Overall yield, 79%.

25-Methyl-25:25-dicarbethoxy-24-homocholane (IV, R′ = Et). A mixture of Na (0·9 g.; 0·039 mole) and diethyl methylmalonate (7·5 ml.; 0·044 mole) was refluxed with dry toluene (50 ml.) until the formation of the sodio derivative was apparently complete (approx. 3 hr.). 24-Iodocholane (5·0 g., 0·011 mole) in dry toluene (30 ml.) was then slowly added from a dropping funnel whilst the mixture was boiled under reflux, addition taking place during 0·5 hr. After boiling for a further 7 hr. the reaction mixture was treated with water and extracted with ether. The extract, after washing with water, drying with Na_2SO_4 and evaporation, gave an oil. This oil was dissolved in a small quantity of pentane and adsorbed on to a column of Al_2O_3 (120 g.). Elution of the column with pentane (400 ml.) resulted in the recovery of crystalline material (2·88 g.) which was composed chiefly of unchanged iodocholane. Elution with pentane/benzene (80% v/v) gave crystalline material (2·04 g.; 37%), recrystallization of which from a little acetone gave *25-methyl-25:25-dicarbethoxy-24-homocholane*, m.p. 64–66°. (Found: C, 76·9; H, 10·6. $C_{32}H_{54}O_4$ requires C, 76·4; H, 10·8%.)

24-Ethoxycholane (VI, R″ = C_2H_5). In an initial condensation, Na (92 mg.) was dissolved in a mixture of diethyl methylmalonate (0·6 ml.) and ethanol (3 ml.). 24-Chlorocholane (1·43 g.) was then added over a period of 15 min., followed by further ethanol (7 ml.). After a total refluxing time of 2·5 hr., treatment with water and ether, followed by washing, drying and evaporation of the ether gave an oil (1·6 g.) which after chromatography in pentane (approx. 200 ml.) on Al_2O_3 (60 g.) gave unchanged chlorocholane (1·0 g.) in the pentane eluates and a second crystalline compound (0·14 g.) in the pentane/benzene (80%, v/v) eluates. Recrystallization of the latter (0·14 g.) from acetone yielded (probably) *24-ethoxycholane*, m.p. 88–90°. (Found: C, 83·4; H, 12·2. $C_{26}H_{46}O$ requires C, 83·4; H, 12·4%.)

In another experiment, condensation of 24-chlorocholane (365 mg.) with diethyl methylmalonate (0·15 ml.) gave, after purification, 25-methyl-25:25-dicarbethoxy-24-homocholane (25 mg.; 5%) as well as unchanged chlorocholane.

24-Methoxycholane (VI, R″ = CH_3). A procedure similar to that described above, but with methanol instead of ethanol, resulted in the isolation of a compound of m.p. 70·5–71·5° which was thought to be 24-methoxycholane (see below).

24-Iodocholane (100 mg.) was refluxed for 2 hr. with methanolic KOH (10 ml. of 4% w/v). Treatment of the reaction product with water, followed by ether extraction and evaporation of the washed and dried ether, gave an oil. Chromatography of this on Al_2O_3 resulted in a recovery of

the iodocholane (32 mg.; 32%) from the pentane eluates and a compound (46 mg.; 58%) from the pentane/benzene (80%, v/v) eluates, which on recrystallization from acetone gave crystals of 24-*methoxycholane*, m.p. 71–71·5°. (Found: C, 82·9; H, 12·5. $C_{25}H_{44}O$ requires C, 83·2; H, 12·3%.) The mixed melting point with the product described above was 71–72°.

Hydrolysis of the ester (IV, R = Et). The above ester (0·6 g.) with ethanol (12 ml.) and aqueous KOH (1·2 ml. of 40%, w/v) was heated in a metal bomb for 2·5 hr. at 120–125°. Dilution with water of the contents of the cooled bomb gave a clear solution which on acidification with HCl yielded a gelatinous solid. Before collection of this the mixture was treated with excess NaCl to ensure complete precipitation. The precipitate was collected by suction on a filter, washed with water and squeezed as dry as possible; it was then dried *in vacuo* over H_2SO_4. The total yield of dried product (IV, R = H) from 2·1 g. of ester was 1·84 g.: no attempt was made at further purification.

Coprostanic acid (V). The above product (50 mg.) was heated in a sublimation apparatus at 0·05–0·02 mm. Hg. At about 165°, decarboxylation occurred and the product changed into a brown oil. This was distilled up to about 200°, forming a nearly colourless 'glass', which was collected and dissolved in methanol. Careful dilution of the warm solution, followed by standing, caused the crystallization of coprostanic acid as long colourless needles (36 mg.; 86% calculated from IV, R′ = Et), which had m.p. 106–108°. The mixed melting point with coprostanic acid (m.p. 103·5–105·5°) from bile salts of Crocodylidae (Haslewood, 1952) was 104–106·5°. $[\alpha]_D^{21°} +28 \pm 1°$ in $CHCl_3$ (*c*, 1·5). (Found: C, 80·6; H, 11·9. Calc. for $C_{27}H_{46}O_2$: C, 80·5; H, 11·5%.) On a larger scale, decarboxylation followed by distillation at up to 280°/0·05 mm. Hg of the acid (IV, R′ = H) (1·84 g.) finally gave coprostanic acid, crystallizing as long white needles (1·25 g.) from aqueous acetone.

The above-mentioned 'glass' (0·1 g.) formed by distillation of IV (R = H) in methanol was treated with excess diazomethane. The solvent was evaporated and the residue, in pentane, was poured on to Al_2O_3 (5 g.). Elution with 8% (v/v) benzene/pentane gave a total of 68 mg. of crystalline material, apparently a single substance. This was *methyl coprostanate*, which crystallized from methanol as colourless needles, m.p. 56–57°. (Found: C, 80·6; H, 11·7. $C_{28}H_{48}O_2$ requires C, 80·7; H, 11·6%.)

Infrared spectra

The infrared spectra were measured by Dr A. R. H. Cole with a Perkin-Elmer Spectrometer, model 12*c*, equipped with a sodium chloride prism, kindly made available by the Chester Beatty Research Institute. A 1 mm. cell was used. Solvents were: 650–1310 cm.$^{-1}$, CS_2; 1310–3200 cm.$^{-1}$, CCl_4. Concentrations in CS_2 were: coprostanic acid, 16·44 mg./ml.; alligator stem acid, 12·64 mg./ml. The CCl_4 solutions were made up using recovered material; the concentrations were not known accurately. The region between 840 and 880 cm.$^{-1}$ was partly obscured by an absorption band of CS_2, and this region is omitted in view of the doubtful accuracy of the spectra.

DISCUSSION

The present work makes clear beyond reasonable doubt the nature of the carbon skeleton and the position of the carboxyl group in derivatives of coprostanic acid. It provides a substance which should be useful as a reference compound in the C_{27} series of bile acids and alcohols.

It might be expected that a malonic ester synthesis of the type described would lead to a mixture racemic with respect to the new asymmetric centre introduced at $C_{(25)}$. Such, however, did not appear to be the case, for the 'natural' and partially synthetic coprostanic acids seemed to be identical in all properties, including optical rotation, which we examined. It is, perhaps, difficult to be absolutely certain on this point, because of the very small quantity of the 'natural' material available for rotation measurements. We have, of course, no direct experience to guide us as to what the general properties of a mixture of substances isomeric at $C_{(25)}$ would be. Comparison with cholanic acid ($[M]_D$, +79°) suggests that in our coprostanic acid ($[M]_D$, +113°), the contribution to $[M]_D$ of the new centre at $C_{(25)}$ is about +34°: a negative value for this in the 25-epimer should have been detected when the rotations were examined.

The method of partial synthesis seems to us to be of special value for the investigation of higher homologues of the C_{24} bile acids and we hope to apply it in other cases.

SUMMARY

1. Cholanic acid ($C_{24}H_{40}O_2$, side chain ; $-CH(CH_3).CH_2.CH_2.COOH$) has been converted by a malonic ester synthesis to coprostan-26-oic acid (coprostanic acid, $C_{27}H_{46}O_2$; side chain $—CH(CH_3).(CH_2)_3.CH(CH_3)COOH$). The intermediates and by-products of this partial synthesis have been isolated and characterized.

2. Coprostanic acid has been shown to be identical with the C_{27} stem acid previously made from the bile salts of Crocodylidae; it should be a useful reference compound.

The authors wish to express their gratitude to Dr A. R. H. Cole (University of Western Australia) for the infrared measurements. They also thank Dr Norman Evers and Messrs Allen and Hanburys Ltd. for a generous gift of cholic acid.

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The Separation and Determination of ^{24}Na and ^{32}P in Animal Tissues

By A. H. ENNOR and H. ROSENBERG
Department of Biochemistry, John Curtin School of Medical Research, The Australian National University, Canberra, A.C.T. Australia

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In experiments employing either ^{24}Na or ^{32}P, the determination of the radioactivity of each isotope in tissues or tissue extracts presents no difficulty, although inaccuracies may arise because of the lack of specificity of methods employed, especially if it is desired to measure the activity of ^{32}P when this is present as inorganic phosphate (cf. Ennor & Rosenberg, 1952*a*). Problems do arise, however, when both isotopes are simultaneously employed, and it is essential to assess the contribution of each to the total activity. For such a differentiation, Hevesy & Rebbe (1940) have taken advantage of the difference in the rates of decay of these isotopes. Thus, since ^{24}Na and ^{32}P have half-lives of 14·8 hr. and 14·3 days respectively, an initial measurement of the radioactivity of a mixture containing both these isotopes, followed by a measurement some days later, will permit the calculation of the contribution which each isotope made to the initial activity of the mixture, but the accuracy of such a procedure will depend upon the proportion in which each isotope was initially present. If the mixture contained roughly equal proportions of each, or an excess of ^{24}Na, such a procedure would be expected to give accurate results. On the other hand, if the radioactivity of ^{32}P present greatly exceeded that of ^{24}Na an error would almost certainly be introduced.

Experiments to be described later (Ennor & Rosenberg, 1952*b*) have employed amounts of ^{24}Na and ^{32}P such that the radioactivity of ^{32}P exceeded that of ^{24}Na by a factor of 5–10 and the present communication is concerned with a method for measuring each isotope accurately.

METHODS AND RESULTS

Principle of the separation. The method to be described depends on the separation of ^{32}P and ^{24}Na by preferential extraction of ^{32}P as a molybdate complex from an aqueous phase into *iso*butanol. ^{24}Na remains in the aqueous phase.

Measurement of radioactivity. In all cases radioactivity was determined as described by Ennor & Rosenberg (1952*a*).

Development of procedure and results

An initial experiment was carried out to determine the maximum amount of inorganic P which could be extracted from a 10 ml. volume without appreciable loss. For this purpose, a sample (10·0 g.) of skeletal muscle was taken from a rabbit which had received intravenously 1 mc. ^{32}P as $NaH_2{}^{32}PO_4$ 24 hr. before. The muscle sample was ashed in a Pt crucible in an electric muffle at about 800°. Complete oxidation of the material to clear inorganic salts was achieved in about 45 min. After cooling, the salts were dissolved in about 0·5 ml. of 10 N-H_2SO_4 and the solution transferred to a 25 ml. graduated flask. The crucible was repeatedly washed with small volumes of N-H_2SO_4 and the washings added to the original solution. When the combined washings reached about 15 ml. (fifteen washes) the radioactivity remaining in the crucible was checked by means of a conventional end-window counter. The counts remaining did not exceed the background count by more than 20 counts/min.

The combined washings were then allowed to stand in a water bath at 100° for 30 min. to hydrolyse pyrophosphate. After cooling to room temperature the volume was adjusted to 25 ml. with N-H_2SO_4. The inorganic P content and the total radioactivity were measured on a 1 : 100 dilution of this solution.

The precise procedure followed was varied somewhat throughout the three experiments, the results of which are given in Table 1.

In Exp. 1 a 1·0 ml. sample of the undiluted solution of inorganic salts was pipetted into a separating funnel, together with 2·0 ml. of 5% (w/v) NaCl, 0·4 ml. 10 N-H_2SO_4 and 4·1 ml. of water. Then 2·5 ml. of 5% (w/v) ammonium molybdate and 20 ml. of *iso*butanol (saturated with water) were injected simultaneously and the flask, after stoppering, was vigorously shaken for 10 sec. The separated aqueous layer was carefully drawn off into a second separating funnel containing 20 ml. of *iso*butanol, which had been previously saturated with water. This flask was then shaken for 10 sec.

Table 1. *Extraction of* ^{32}P *from aqueous phase with* isobutanol

	Exp. 1	Exp. 2	Exp. 3
Volume of sample (ml.)	1·0	5·0	5·0
Total radioactivity added (counts/min.)	5830	29 150	29 150
Inorganic P added (mg.)	0·725	3·62	3·62
Volume and concentration of ammonium molybdate added	2·5 ml. of 5% (w/v)	2·5 ml. of 5% (w/v)	2·5 ml. of 20% (w/v)
Total radioactivity (counts /min.) found in aqueous phase after extraction with *iso*butanol	8	15 590	40

Table 2. *The insolubility of* ^{24}Na *in* iso*butanol*

Radioactivity of aqueous phase (counts/min.)				Radioactivity in *iso*butanol (counts/min.)	
Aqueous layer*	Wash 1†	Wash 2†	Total	In presence of 2·5 ml. 20% (w/v) ammonium molybdate	In absence of ammonium molybdate
1750	125	5	1880	122	0

* Aqueous layer refers to the initial aqueous solution after extraction with *iso*butanol.

† Wash 1 and wash 2 refer respectively to the first and second volumes of N-H_2SO_4 with which the *iso*butanol was washed after the initial aqueous solution had been extracted and run off. All phases were made to 25 ml. before counting For further details see text.

and the aqueous layer collected in a 25 ml. volumetric flask. The first *iso*butanol extract was then shaken with 10 ml. of N-H_2SO_4 and this, after separation, was run into the second funnel where the aqueous layer was washed with *iso*butanol. This washed aqueous layer was then combined with the first and made to 25 ml. by the addition of water previously saturated with *iso*butanol. The solution, after mixing, was finally filtered through Whatman filter paper no. 1, and its activity measured. The use of *iso*butanol saturated with water was adopted to prevent the diminution in volume of the aqueous phase, due to the solubility of water in the solvent. Water, saturated with *iso*butanol, was used to adjust volumes to 25 ml. in order to avoid solution of the very fine drops of *iso*butanol which are commonly present in the aqueous phase. A final filtration through paper removes all such contamination.

Exps. 2 and 3 followed similar lines, except that 5·0 ml. samples were taken and in Exp. 3 the use of 20% (w/v) ammonium molybdate was adopted in place of the 5% (w/v) solution which had been used before. The volume of the aqueous phase was 10 ml.

The results (Table 1) show that in Exp. 1, in the presence of 0·725 mg. of inorganic P, the efficiency of the extraction procedure was such that only 8 counts/min. out of a total of 5830 counts/min. were not extracted and thus appeared in the aqueous phase. In Exp. 2, when a 5·0 ml. sample was taken, the amount of inorganic P present (3·62 mg.) was in excess of that which could be combined with molybdic acid to form phosphomolybdic acid and, as a result, 15 590 counts/min. were found in the aqueous phase out of a total of 29 150 counts/min., which were originally present. That this is the correct explanation is proven by Exp. 3, where the amount of ammonium molybdate present was increased by a factor of 4. In this experiment only 40 counts/min. were found in the aqueous phase.

It is thus clear that, if the procedure adopted in Exp. 3 is followed, when relatively large amounts of inorganic P are present, the efficiency of the extraction procedure is such that it may be anticipated that 99·90% of the inorganic P will be extracted into the *iso*butanol.

The number of counts (40) found in the aqueous phase of Exp. 3 is that which would be expected on stoicheiometric grounds (cf. 8 counts/min. in Exp. 1). This led to the belief that these counts may have been due to the presence of a minute amount of inorganic pyrophosphate which had escaped hydrolysis and which would not react with molybdic acid and therefore would not be extracted by the *iso*butanol.

As a test of this hypothesis the aqueous layer from Exp. 3 was subjected to further hydrolysis for a period of 30 min. at 100° and re-extracted with *iso*butanol. After this treatment no radioactivity could be detected in the aqueous layer. It may, therefore, be concluded that a completely quantitative separation is readily achieved.

The successful application of this method to experiments in which ^{32}P and ^{24}Na are simultaneously employed depends also upon the retention of ^{24}Na in the aqueous phase. To test the possibility of ^{24}Na entering the alcohol phase, additional experiments have been carried out using similar techniques as were used in Exp. 3 above. In these experiments a sample containing ^{24}Na as ^{24}NaCl was pipetted into the separating funnels together with the usual reagents. In one case ammonium molybdate was present and in the other it was omitted, but in no case was inorganic P added.

In these experiments, since it was necessary to determine the radioactivity in each aqueous phase after the washing of the *iso*butanol layer, neither the aqueous layer nor the two washes were combined. Each was made to 25 ml. and, together with the *iso*butanol phase, checked for radioactivity.

As far as the aqueous phase is concerned (Table 2), it is seen that the bulk of the ^{24}Na is removed when the original solution is run off after extraction. Of the radioactivity which remains in the separating funnel, 125 counts/min. are removed in the first wash and 5 in the second. It will also be noted that 122 counts/min. were detected in the *iso*butanol when extraction was carried out in the presence of molybdate. When, however, molybdate was omitted no radioactivity could be detected. This finding is suggestive of a contaminant in the ^{24}NaCl which reacts with ammonium molybdate to form a complex which is preferentially soluble in *iso*butanol, but which, in the absence of molybdate, is retained in the aqueous layer.

Table 3. *Decay of radioactivity in* iso*butanol layer*

(For details see text.)

Date of counting	Time of counting	Interval	Counts/min.
5. iii. 52	14.30 hr.	0	104
5. iii. 52	16.50 hr.	2 hr.	103
6. iii. 52	06.50 hr.	16 hr.	103
7. iii. 52	12.10 hr.	46 hr.	91
18. iii. 52	16.30 hr.	13 days 2 hr.	52

The chemical behaviour of this contaminant, together with the decay rate (Table 3), show that it is ^{32}P and that ^{24}NaCl, as supplied by the Atomic Energy Research Establishment, Harwell, England, is contaminated with ^{32}P, which is present as inorganic P. It is, in fact, formed from the Cl of the NaCl by the following reaction: ^{35}Cl (n, α) ^{32}P.

The results of a final experiment in which the two isotopes were quantitatively separated from a mixture of each in plasma and muscle is of interest (Table 4).

Table 4. *Separation of* ^{24}Na *and* ^{32}P *from samples of plasma and muscle*

(For treatment of animal, see text. All results are expressed as radioactivity counts/min./ml. of plasma or/g. of muscle.)

		Aqueous layer containing ^{24}Na		
	*iso*Butanol layer containing ^{32}P	Observed		Calculated*
		0 time	0+108 hr.	0+108 hr.
Plasma	139 700	1750	11	11
Muscle	9 450	1010	9	7

* Based on the theoretical counts/min. expected, assuming that the counts/min. observed at 0 time were due entirely to ^{24}Na.

In this experiment a well fed rabbit (2·4 kg.) was injected intravenously with approximately 15 μc. of ^{24}Na as $^{24}NaCl$ and, 25 min. later, with approximately 1 mc. ^{32}P as $NaH_2{}^{32}PO_4$. The animal was killed 5 min. later and blood collected for separation of plasma. A sample (1 ml.) of plasma was ashed in a Pt crucible at 800° as was done with the muscle samples described above. The inorganic residue was treated in the same way and made to the same volume. The muscle sample was treated as before. Samples from the solutions of inorganic salts from plasma and muscle were then subjected to the extraction procedure, as outlined in Exp. 3, and the radioactivity present in the aqueous phase and in the *iso*butanol determined. It is clear that excellent separation of the two isotopes had been achieved in both cases.

DISCUSSION

The foregoing experimental results indicate that the extraction procedure outlined in Exp. 3 is sufficiently well designed to permit the quantitative measurement and separation of ^{24}Na and ^{32}P from a mixture of each, even though the radioactivity of the ^{32}P present considerably exceeds that of ^{24}Na. The accuracy with which such a separation may be achieved depends upon several factors, the most important of which are: (1) the completeness of the conversion of inorganic pyrophosphate to inorganic orthophosphate. To ensure this, it is apparently necessary to keep the acid solutions of the ashed inorganic salts at 100° for 1 hr.; (2) the maintenance of a sufficient excess of ammonium molybdate to allow the complete transformation of inorganic P to phosphomolybdic acid in which form the ^{32}P may be extracted into *iso*butanol; (3) the speed with which the first-formed phosphomolybdic acid is extracted by the *iso*butanol. In this connexion it should be remembered that when several mg. of inorganic P are present it is essential that the ammonium molybdate and the *iso*butanol be added simultaneously. If any delay in the addition of the *iso*butanol occurs then the phosphomolybdic acid, which forms very rapidly, is formed in such amounts as to exceed the solubility product of the complex in the aqueous phase and precipitation occurs. In the wet solid state the compound is insoluble in *iso*butanol.

Whilst it is clearly advantageous to eliminate all ^{32}P in the aqueous phase and to count only ^{24}Na in this phase, it should be remembered that if ^{32}P does appear it can only constitute a small fraction of the total and in any event may be readily corrected for on the basis of results obtained by determination of the residual radioactivity some days later.

The ^{32}P contaminant which was detected in the sample of ^{24}Na was of such an order that when the experiments were commenced the radioactivity due to ^{32}P represented about 7% of the total. Clearly such a contamination could lead to erroneous interpretation of results obtained in *in vivo* experiments because of the different distribution of P and Na throughout the body fluids and tissues. However, it should be remembered that the sample of ^{24}Na employed in the present experiments was used 5 days after leaving Harwell, so that, on the basis of the half lives of both ^{32}P and ^{24}Na, this contamination 5 days earlier would have amounted to about 0·03% of the total. Such a contaminant is negligible at the source of the material, but clearly may be sufficiently large to vitiate results if undetected and used some distance away from the source, e.g. in Australia. It can be avoided by obtaining ^{24}Na from the irradiation of $NaHCO_3$ instead of NaCl.

SUMMARY

A method for the quantitative estimation and separation of ^{32}P and ^{24}Na from a mixture of each in body fluids and tissues is described. ^{32}P is extracted as a molybdate complex into *iso*butanol, ^{24}Na remains in the aqueous phase.

It is a pleasure to express our thanks to Dr C. E. Eddy, Director, Commonwealth X-ray and Radium Laboratory, Melbourne, for the organization and supply of the isotopes used in this study.

Our grateful thanks are also due to Mr G. P. N. Watt, Chairman of the Australian National Airlines Commission, and to Mr C. O. Turner, General Manager, Qantas Empire Airways, for their generosity in transporting freight-free the ^{24}Na used.

We are also grateful to Dr F. G. Morgan, Director of the Commonwealth Serum Laboratories and to the Director General of Health, for a generous provision of laboratory space pending the establishment of this Department in Canberra, and to Miss J. D'Alton for skilled technical assistance.

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Phosphorus Compounds in the Cell

2. THE SEPARATION BY IONOPHORESIS ON PAPER OF THE CONSTITUENT NUCLEOTIDES OF RIBONUCLEIC ACID

By J. N. DAVIDSON and R. M. S. SMELLIE
Department of Biochemistry, The University of Glasgow

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In the first paper in this series (Davidson, Frazer & Hutchison, 1951) it was emphasized that in experiments on the incorporation of radioactive phosphorus (^{32}P) into the nucleic acids and their derivatives in the cell, it was essential, in order to obtain true specific activities, to separate the nucleotide fractions in a form free from all traces of highly active contaminants. The procedure of ionophoresis of nucleotides now to be described is eminently suitable for this purpose.

The separation of ribonucleotides by ion-exchange chromatography on columns has been described by Cohn (1949, 1950) and by Cohn & Carter (1950), and a chromatographic separation on filter paper has been described by Magasanik, Vischer, Doniger, Elson & Chargaff (1950), by Carter (1950), by Markham & Smith (1951*a*) and by Boulanger & Montreuil (1951*a*, *b*).

While these methods have much to commend them, they nevertheless suffer from certain disadvantages. In the method of Magasanik *et al.* (1950), for example, guanylic and uridylic acids share the same position on the chromatogram, while in Carter's (1950) and Markham & Smith's (1951*a*) procedure, the pyrimidine nucleotides share a spot. It has therefore occurred to us that a method utilizing the ionophoretic mobility of nucleotides might prove satisfactory, and the technique of ionophoresis as originally described by Consden, Gordon & Martin (1946) has accordingly been adapted for nucleotide separation. A preliminary account of this work has already been given (Smellie & Davidson, 1951).

EXPERIMENTAL

Initial experiments using silica gel as the medium for ionophoresis were not successful. Agar gel, as used by Gordon, Keil, Sebesta, Knessl & Sorm (1950) in the apparatus of Consden *et al.* (1946), was more satisfactory. The position of the nucleotides after separation was established by inspecting the surface of the gel in light from an ultraviolet lamp fitted with a filter, as described by Holiday & Johnson (1949). Elution was not attempted, but the areas of gel shown to contain nucleotides were cut out and the excised portions dissolved in water for the determination of the ultraviolet absorption spectra of the nucleotides. The agar solution containing the nucleotides was then digested for the determination of P.

This method, while moderately satisfactory, is rather cumbersome and has been superseded by the use of the method of ionophoresis on paper described by Durrum (1950) for amino-acids. The procedure is as follows: an accurately measured volume of the material to be analysed, containing about 80 μg. P, is applied in a narrow band about 3·5 cm. long, about 5 cm. from one end of a strip of Whatman 3 MM paper, 72 × 7 cm. The application is made from an Agla micrometer syringe (Burroughes Wellcome Ltd.), and by holding the paper in a stream of warm air it is not difficult to spot up to 500 μl. Excessive heat must be avoided since it causes spontaneous formation of isomeric nucleotides. The strip is then moistened with 0·02 M-citric acid/trisodium citrate buffer, pH 3·5 (obtained by a 50-fold dilution of a mixture of 3 vol. M-citric acid and 1 vol M-trisodium citrate) and suspended over a glass rod so that the two ends are immersed to a depth of 1 cm. in two separate dishes containing about 0·5 l. of the same buffer. Carbon electrodes placed in each dish are connected to a source of direct current so that the anode is in the dish further away from the spot on the paper. The direct current supply consists of a full-wave valve rectifier capable of a smoothed output of 600 V. at 300 ma., which on a small load actually delivers about 800 V. The output is controlled by means of a Variac transformer in the input side which makes possible a complete range of 100–800 V.

Complete separation of adenylic, cytidylic, guanylic and uridylic acids is obtained in amounts of the order of 200–300 μg. of each by running at a potential gradient of 11 V./cm. for 18 hr. The apparatus is covered during the run with a glass case to minimize evaporation, and although it is possible to obtain the same separation in a shorter time by increasing the applied voltage, this procedure results in increased evaporation so that water condenses on the case and is liable to drip on to the paper. The apparatus is of such a size that several papers can be used simultaneously.

On completion of the run, the paper is dried by an infrared lamp, the bands located in ultraviolet light according to the procedure of Holiday & Johnson (1949) and marked lightly in pencil. If a permanent record is desired a photograph may be made by the method of Markham & Smith (1949). The bands are then cut out and the nucleotides eluted by the method of Consden, Gordon & Martin (1947) into graduated Pyrex tubes. The volume of the eluate is adjusted to 5 ml. with distilled water, sufficient 5 N-HCl having been added to give a final concentration of 0·1 N. The ultraviolet absorption spectrum of each eluate is determined on the Beckman Model DU spectrophotometer or a Unicam SP 500 quartz spectrophotometer. Identification is carried out first from

the positions of the bands, and secondly by relating the ultraviolet absorption spectra of the eluates to those of the pure nucleotides at the same pH.

As a further check on the identity of the nucleotides, occasional samples of the eluates have been hydrolysed with $HClO_4$ according to the method of Marshak & Vogel (1950, 1951) and the bases separated by paper chromatography by the procedure of Wyatt (1951) and characterized by their ultraviolet absorption. In every case only the appropriate base has been found. These separations were kindly carried out by Dr G. Crosbie.

P was estimated in the eluates by a micromodification of the method of Allen (1940).

The method of ionophoresis has been applied to the analysis of various specimens of ribonucleic acid (RNA). A commercial preparation of yeast RNA made by British Drug Houses Ltd. was purified by the method of Smith & Markham (1950) followed by precipitation from glacial acetic acid. Liver RNA was isolated by the method of Davidson *et al.* (1951). The hydrolysis of these purified materials was carried out by incubation with 0·3 N-KOH for *18 hr. at 37°. The volume of alkali used was 1 ml./5 mg. of RNA. The alkaline digest was brought to pH 1 in the cold by the addition of 60% (w/w) $HClO_4$, insoluble $KClO_4$ was centrifuged out and washed, and the supernatant fluid and washings adjusted to pH 4 with N-KOH. P was estimated in one portion of this solution while a second portion was applied to the paper in amounts corresponding to approximately 80 μg. P.

Since the isolation procedure of Davidson *et al.* (1951) is tedious and time-consuming, the following modification has been adopted. A homogenate of liver tissue was extracted with trichloroacetic acid and lipid solvents as described by Davidson *et al.* (1951). The dry powder so obtained was extracted three times for 1 hr. each with 10% (w/v) NaCl solution at 100°. The combined extracts were treated with 2 vol. ethanol, and the precipitate of sodium nucleate was washed with ethanol and ether, and dried. The dry powder in the proportion of 20 mg./ml. was incubated with 0·3 N-KOH at 37° for 18 hr. and brought to pH 1 in the cold by addition of 60% $HClO_4$ as described above. The precipitate of deoxyribonucleic acid (DNA), protein and $KClO_4$ was spun down and removed and the acid extract applied to the paper.

This application of the Schmidt & Thannhauser (1945) principle was found to give material, which, while not as pure as an isolated nucleic acid, was nevertheless eminently suitable for ionophoresis. For reasons to be discussed in the next paper the use of the RNA fraction obtained by application of the Schmidt & Thannhauser (1945) method to the tissue itself is complicated by the presence of interfering factors for which special allowance must be made.

In preliminary tests samples of all four purified nucleotides manufactured by Nutritional Biochemicals Inc. were used. Guanylic and cytidylic acids supplied by British Drug Houses Ltd., and adenylic acid by Ernst Bischoff Co. Inc. were also employed.

RESULTS

The separation of pure nucleotides by ionophoresis is illustrated in Fig. 1, which is a photograph taken in ultraviolet light by the method of Markham & Smith (1949) of a sheet of paper to which all four nucleotides had been applied separately and as a mixture, and subsequently subjected to ionophoresis. It is obvious that adequate separation can be achieved by this method and that the nucleotides separate in the order uridylic acid, guanylic acid adenylic acid and cytidylic acid in decreasing order of mobility. From Table 1 it is seen that the recovery of pure, single nucleotides applied to filter paper, run and eluted is adequate; Table 2 shows that the recoveries from a mixture of the four pure nucleotides are also satisfactory.

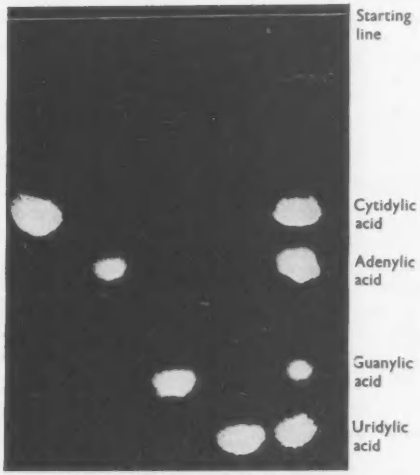

Fig. 1. Ionophoretic runs of single nucleotides and of a mixture of nucleotides. The paper has been photographed on Ilford reflex document paper by ultraviolet light according to the method of Markham & Smith (1949).

Table 1. *Recovery of pure nucleotides after ionophoresis*

(Each nucleotide was applied to the paper singly, run and eluted.)

Nucleotide	P applied (μg.)	P recovered (μg.)	Recovery (%)
Cytidylic acid	25·0	24·6	98·5
	19·2	19·0	99·0
	25·4	24·1	95·5
Adenylic acid	20·7	20·0	97·0
	20·6	20·1	97·5
	20·7	19·8	96·0
Guanylic acid*	19·6	19·0	97·0
	19·6	19·2	98·0
	10·0	9·4	94·0
Uridylic acid	24·7	23·7	96·5
	23·2	22·8	98·5
	23·3	23·7	101·0

* Previously purified by ionophoresis.

Table 2. *Recovery of pure nucleotides from a mixture separated by ionophoresis on paper*

Nucleotide	P applied (μg.)	P recovered (μg.)	Recovery (%)
Cytidylic acid	19·2	18·3	95·5
	25·0	24·6	98·5
	25·4	25·0	98·5
Adenylic acid	20·6	19·4	94·5
	20·7	19·6	94·5
	20·7	19·7	95·0
Guanylic acid*	10·0	10·2	102·0
	10·0	9·9	99·0
	10·0	9·8	98·0
Uridylic acid	23·2	22·0	95·0
	24·7	25·4	102·5
	23·3	21·8	93·5

* Previously purified by ionophoresis.

Table 3. *Analysis of a mixture of pure nucleotides (a) by ionophoresis and (b) by chromatography of the bases derived from the nucleotides by perchloric acid hydrolysis and separated by the method of Wyatt (1951)*

(The results are expressed as molar ratios relative to adenylic acid as 10.)

Nucleotide	Ionophoresis	Chromatography
Cytidylic acid	10·8	10·8
Adenylic acid	10·0	10·0
Guanylic acid*	9·0	9·3
Uridylic acid	13·4	12·3

* A correction has been made for 4% free guanine present in the sample of guanylic acid used.

A check on the validity of the ionophoretic procedure is shown in Table 3 which gives the results of analysis of a mixture of pure nucleotides by two different methods. One portion of the solution was submitted to ionophoresis and phosphorus determined in the eluted nucleotides. A second portion of the solution was evaporated to dryness, digested with perchloric acid, the bases separated by paper chromatography (Wyatt, 1951) and determined by ultraviolet absorption measurements. There is, in general, good agreement except for uridylic acid for which it was invariably found that the figure obtained by ionophoresis was slightly higher than that found by chromatography.

Marrian, Spicer, Balis & Brown (1951) have shown that, when cytidylic acid is incubated in N alkali at 37° for 18 hr., partial deamination occurs with the formation of uridylic acid. This observation has been confirmed by taking portions of cytidylic acid which had been incubated for 18 hr. in N-KOH at 37° and 25° and in 0·3N-KOH at 37° and submitting them to ionophoresis. Only after incubation in N-KOH at 37° was any detectable amount of uridylic acid produced. The quantity involved was about 10% of the original cytidylic acid.

A mixture containing all four nucleotides was then divided into four portions, one of which was maintained as a control while the others were incubated in N-KOH at 37° and at 25° and in 0·3N-KOH at 37°, respectively. The pH of each fraction was adjusted to 4 with 60% percholoric acid, the precipitate of potassium perchlorate was centrifuged out and portions of the supernatant fluid applied to paper in the usual way. After ionophoresis the nucleotides were eluted and phosphorus determined in each eluate. The results of this experiment are illustrated in Table 4 which shows that no deamination occurs after incubation in 0·3N-KOH at 37° although deamination is appreciable in N-KOH at 37°.

Table 4. *Analysis of a mixture of four nucleotides before and after incubation in* 0·3N-KOH *and* N-KOH *at 25° and 37°*

(The mixture after acidification was submitted to ionophoresis and P determination on the eluates. The results are expressed as molar ratios relative to adenylic acid as 10.)

Nucleotide	Un-incubated	0·3N-KOH at 37°	N-KOH at 25°	N-KOH at 37°
Cytidylic acid	11·0	10·8	10·3	9·9
Adenylic acid	10·0	10·0	10·0	10·0
Guanylic acid	9·1	9·0	9·0	9·4
Uridylic acid	13·1	13·4	13·0	15·0
P recovered (%)	98·0	98·5	100·0	92·0

The method of ionophoresis was then applied to the separation of the ribonucleotides obtained by digestion of specimens of purified RNA with 0·3N-KOH at 37°. The extent of liberation of acid-soluble phosphates from RNA by this procedure is illustrated in Table 5, from which it is clear that all

Table 5. *Liberation of acid-soluble phosphates from a specimen of yeast ribonucleic acid by alkaline hydrolysis followed by acidification*

(In each case 37·5 mg. RNA were incubated with 5 ml. 0·3N-KOH at 37° for 18 hr.)

Total P in alkaline digest (μg.)	Total P in acid supernatant (μg.)	Recovery (%)
3150	3070	97·5
3128	3025	96·7
3143	3035	96·6

the phosphorus of the RNA is liberated in the form of acid-soluble phosphates. The fact that only about 97% of the RNAP is recovered in the acid supernatant is almost certainly due to small losses incurred in the process of acidifying, removing the precipitated potassium perchlorate and washing the precipitate.

Specimens of isolated nucleic acid from yeast and from rat liver were then submitted to analysis by ionophoresis of the nucleotides obtained by alkaline incubation, with subsequent estimation of phosphorus in the eluates, and also by paper chromatography of the bases liberated by digestion of the

Table 6. *Analysis of purified yeast and liver ribonucleic acids (a) by ionophoresis of the nucleotides obtained by alkaline incubation with subsequent estimation of* P *in the eluates and (b) by paper chromatography of the bases after digestion with perchloric acid*

(The results are expressed as molar ratios with respect to adenylic acid or adenine as 10.)

	Yeast RNA		Rat-liver RNA	
Nucleotide	(*a*)	(*b*)	(*a*)	(*b*)
Cytidylic acid	8·8	8·1	14·5	13·9
Adenylic acid	10·0	10·0	10·0	10·0
Guanylic acid	12·4	11·5	17·6	17·5
Uridylic acid	10·4	7·9	10·1	8·1
P recovered (%)	93·5	—	93·8	—

RNA with perchloric acid. The results are shown in Table 6, while an ultraviolet photograph of the strip of paper used in the separation of the nucleotides in the sample of yeast RNA is shown in Fig. 2. The relative amounts of the four nucleotides are similar to those quoted by other workers (Chargaff, 1950; Chargaff *et al.* 1950; Smith & Markham, 1950). The liver nucleic acid differs from the yeast RNA in its higher contents of cytidylic and guanylic acids. In each case, however, the figure obtained for uridylic acid was appreciably higher by the ionophoresis of nucleotides than by chromatography of the bases.

The results of the analysis of specimens of RNA obtained by the modified isolation procedure in which the dry tissue powder is extracted with 10% sodium chloride solution and the ribonucleic acid so obtained submitted to alkaline hydrolysis are shown in Table 7.

The figures obtained by this method are very similar to those shown in Table 6 for isolated rat-liver RNA. Rat and rabbit livers give values of the same order, while the results obtained for rat-liver mitochondria and microsomes prepared by the method of Schneider (1948) do not differ appreciably from those for whole liver tissue.

DISCUSSION

In any analytical procedure designed for the separation of the constituents of the nucleic acids, the chief difficulty is to establish a method of hydrolysis which will bring about adequate

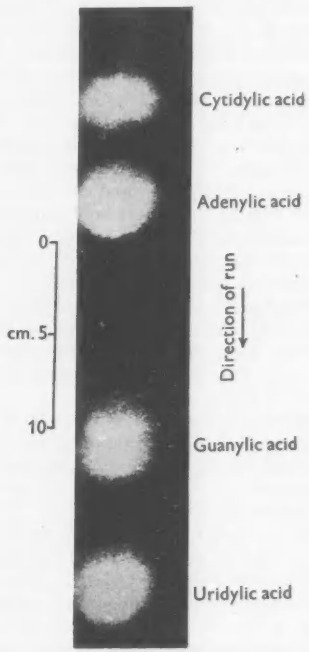

Fig. 2. Ultraviolet print showing separation by ionophoresis on paper of the four constituent nucleotides obtained from yeast RNA by alkaline digestion.

cleavage without partial destruction of any component or interconversion of components. While the separation of the purine and pyrimidine bases by chromatography is straightforward with suitable solvents, the complete and quantitative hydrolysis of a nucleic acid to the bases is more difficult. Hydrolysis of the RNA to purine bases and pyrimidine nucleotides with N-hydrochloric acid also

Table 7. *Composition of ribonucleic acid from liver tissue as determined by ionophoresis of the ribonucleotide fraction obtained by alkaline incubation of the nucleic acid extracted with* 10% NaCl

(The results are expressed relative to adenylic acid as 10.)

Nucleotide	Rat liver 1	Rat liver 2	Rabbit liver	Rat-liver mitochondria	Rat-liver microsomes
Cytidylic acid	13·9	14·3	14·6	13·4	14·8
Adenylic acid	10·0	10·0	10·0	10·0	10·0
Guanylic acid	17·5	17·6	16·9	17·3	18·2
Uridylic acid	10·9	10·8	10·3	10·8	11·1

appears to give rise to difficulties since Markham & Smith (1951*a*) have found it necessary to apply a correction for partial hydrolysis of pyrimidine nucleotides. Hydrolysis of RNA by alkali to the constituent nucleotides on the other hand is simple and quantitative and involves no destruction of base, provided that conditions are mild enough to avoid deamination of cytidylic acid. The quantitative analysis of a mixture of nucleotides, however, presents certain difficulties to which, it is now clear, the procedure of ionophoresis on paper offers a convenient solution. Ionophoresis is simple and rapid to carry out and has the advantage that the result can be quickly demonstrated by visual inspection in ultraviolet light. Chromatography with certain solvents, e.g. phenol as used by Boulanger & Montreuil (1951*a*, *b*) renders such inspection impossible, and in any case most solvent systems used in the chromatography of nucleotides on paper fail to give adequate separation of at least two of them. The electrophoretic separation of nucleotides under conditions rather different from ours has been used by Markham & Smith (1951*b*) for the separation of the di- and tri-nucleotides obtained by partial hydrolysis of RNA.

The separation of all the known ribonucleotides can be achieved by chromatography on columns of ion-exchange resins, but ionophoresis on paper is more convenient to carry out and is more suitable for small quantities.

In the analysis of samples of purified RNA the results for the base ratios obtained by ionophoresis are similar to those obtained by other workers using paper chromatography (Magasanik *et al.* 1950; Markham & Smith, 1951*a*) or column chromatography (Volkin & Carter, 1951). When the results of ionophoresis of nucleotides are compared for the same specimen of RNA with those obtained by paper chromatography (Table 6) after hydrolysis to the bases with perchloric acid by Marshak & Vogel's (1951) method, a higher figure for uridylic acid is invariably given by the former procedure. The same discrepancy is found with mixtures of pure nucleotides and is probably due to some destruction of uracil by the perchloric acid. Recovery of uridylic acid based on phosphorus estimations and ultraviolet determinations on eluates from runs has shown that the amount of uridylic acid recovered is the same and accounts for all the uridylic acid applied to the paper. This problem will be discussed in greater detail in a separate communication.

Although the hydrolysis of RNA to nucleotides yields acid-soluble phosphates quantitatively, the nucleotides separated by ionophoresis account for not more than 94% of the total phosphorus in the hydrolysate (Table 6). While some of the remaining 6% can no doubt be accounted for by the slight experimental losses inherent in any such procedure as this, the presence of small amounts of non-nucleotide phosphorus cannot be excluded. Experiments with isolated RNA labelled with ^{32}P suggest that such, in fact, is the case.

Although the procedure of ionophoresis of nucleotides is eminently suitable for the analysis of purified specimens of RNA, the isolation of RNA by such procedures as that described by Davidson *et al.* (1951) is a lengthy process. The modified procedure described above in which the nucleic acids are extracted from the tissue powder with 10% sodium chloride and are subsequently submitted to a Schmidt-Thannhauser separation yields satisfactory results in a much shorter time. When the usual Schmidt & Thannhauser (1945) separation procedure is employed in which the dry lipid-free powder is incubated in alkali to hydrolyse RNA, the ribonucleotide fraction obtained by acidification of the alkaline digest is found to contain several other phosphate derivatives which greatly complicate the picture. They are discussed in the following paper.

SUMMARY

1. A method is described for the separation of ribonucleotides by ionophoresis on paper.

2. It can be applied to the analysis of a ribonucleic acid after alkaline digestion or to the ribonucleotide fraction obtained from a tissue by a modification of the method of Schmidt & Thannhauser.

We wish to thank Mr D. R. S. Cameron and Mr J. W. Sommerville for valuable technical assistance in the course of this work. Our grateful thanks are also due to the Medical Research Council and the British Empire Cancer Campaign for expenses grants, and to Messrs Smith, Kline and French of Philadelphia for gifts of pure nucleotides.

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Phosphorus Compounds in the Cell

3. THE INCORPORATION OF RADIOACTIVE PHOSPHORUS INTO THE RIBONUCLEOTIDE FRACTION OF LIVER TISSUE

BY J. N. DAVIDSON AND R. M. S. SMELLIE
Department of Biochemistry, The University of Glasgow

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In previous work on the distribution of radioactive phosphorus among the protein-bound phosphorus fractions of animal tissues (Davidson, Frazer & Hutchison, 1951) it has been pointed out that the removal of the last traces of contaminating acid-soluble phosphate from such fractions is an extremely difficult process, and the conclusion has been reached that it is unjustifiable to assume that the specific activities of the nucleic acid fractions obtained by the separation procedure of Schmidt & Thannhauser (1945) represent the true activities of the nucleic acids. In such experiments it is essential to eliminate the possibility of the presence of contaminating inorganic phosphate by a rigorous purification of each fraction. In the Schmidt-Thannhauser procedure the fraction most susceptible to interference is that containing the acid-soluble nucleotides formed from ribonucleic acid (RNA) by alkaline hydrolysis and subsequent acidification. A method for the separation and purification of these nucleotides in small quantities would therefore be of great value. The method of ionophoresis described by Smellie & Davidson (1951) and Davidson & Smellie (1952) has proved satisfactory for the purpose and its application will now be described.

METHODS

The technique of ionophoresis was employed as described in the previous paper (Davidson & Smellie, 1952).

Two separate methods were adopted for preparing material for ionophoresis.

Procedure 1. A modification of the Schmidt & Thannhauser (1945) method for the separation and estimation of nucleic acids was used. The tissue homogenate was extracted with trichloroacetic acid (TCA) and with lipid solvents to remove acid-soluble P and lipid P as described by Davidson *et al.* (1951). Water was then added in the proportion of 1 ml. to 100 mg. of the dry lipid-free residue and 5 N-KOH to give a final concentration of 0·3 N. The mixture was then incubated at 37° for 18 hr. The resulting digest was brought to pH 1 with 60% (w/w) $HClO_4$ and the precipitate of protein, deoxyribonucleic acid (DNA) and $KClO_4$ centrifuged down, the supernatant fluid plus washings being used for ionophoresis as the fraction A_2S of Davidson *et al.* (1951) after adjusting the pH to 4.

Procedure 2. Since the isolation of RNA from animal tissues by the method of Davidson *et al.* (1951) is tedious and time-consuming, the following modification was adopted. The tissue homogenate was extracted with TCA and lipid solvents as described in procedure 1. The residue was extracted three times for 1 hr. each at 100° with 10% (w/v) NaCl. The extracts were pooled and treated with 2 vol. ethanol. The precipitate so formed was washed with ethanol and ether and dried. It was hydrolysed to mononucleotides by digestion with 0·3 N-KOH for 18 hr., using 1 ml. alkali/20 mg. powder. The alkaline digest was brought to pH 1 by adding 60% $HClO_4$. DNA, protein and $KClO_4$ were centrifuged out and washed, and the supernatant fluid and washings adjusted to pH 4 with N-KOH.

Radioactive phosphate supplied by the Atomic Energy Research Establishment, Harwell, as H_3PO_4 was submitted to 10 min. hydrolysis with N-HCl before use to destroy any traces of pyro- or poly-phosphates.

The general procedure with radioactive material was that of Davidson *et al.* (1951). ^{32}P was administered subcutaneously to rats in doses of 50 μc./100 g. body weight, and the animals killed 2 or 24 hr. later. The livers were perfused with saline, excised, homogenized in saline in an M.S.E. Nelco Blendor and submitted to procedures 1 and 2 above. Papers for ionophoresis were usually set up in duplicate or triplicate. One set was photographed in ultraviolet light according to the method of Markham & Smith (1949) and then left in contact with Kodak Industrex Type *D* X-ray film for 2 weeks for the preparation of autoradiographs. A second set of papers was cut up and eluted, the eluates being used for the determination of P and radioactivity.

RESULTS

Several authors (Marshak & Vogel, 1950; Jeener & Szarfarz, 1950) have recently stressed the difficulties of removing traces of contaminating ^{32}P from the nucleotide-containing fraction of tissues, and Davidson *et al.* (1951) have demonstrated that, if a small amount of inorganic radioactive phosphate is added to a homogenate of non-radioactive rat liver along with TCA, there is still considerable activity in the ribonucleotide fraction obtained by the method of Schmidt & Thannhauser (1945) even after exceedingly exhaustive washing of the tissue with TCA and with TCA containing phosphate.

Experiments have therefore been carried out to investigate the efficiency of ionophoresis in removing such contaminating ^{32}P. Small amounts of inorganic radioactive phosphate were added to a spot of mixed nucleotides on paper and the mixture submitted to ionophoresis. The progress of the inorganic phosphate band was followed by means of a 'Panax' monitor set, and ionophoresis was generally continued until this band was approaching the end of the paper. The position of the band was obtained, after drying the paper, by moving the monitoring counter slowly over its surface. It was found that all the activity was located in one well defined area, and this was marked in pencil. On subsequent examination of the paper in ultraviolet light, the radioactive region proved to be well clear of the four nucleotides. When the separate nucleotides were eluted the amount of contaminating ^{32}P as determined in a 20th Century Electronics Type M6 liquid counter was found to be negligible.

In another experiment, a small amount of radioactive phosphorus was added to the paper at the starting point along with a volume (containing about 80 μg. phosphorus) of a Schmidt-Thannhauser nucleotide fraction prepared from non-radioactive rat liver. After ionophoresis for 6 hr. at 14 V./cm., the paper was dried and the ultraviolet-absorbing areas marked in pencil. When the 'Panax' monitor was moved slowly over the paper, the only area which exhibited radioactivity was found to lie well ahead of the nucleotides, and to coincide with component *A* (see below). Determination of the activity of the nucleotides indicated negligible contamination from added ^{32}P.

In a third experiment 0·88 μc. ^{32}P was added to a homogenate of 4 g. rat liver along with TCA as had been done by Davidson *et al.* (1951). The precipitated material was washed four times with ice-cold 10% (w/v) TCA and subjected to the usual modified Schmidt-Thannhauser procedure (procedure 1), the alkaline digest being precipitated with perchloric acid. The ribonucleotides separated by ionophoresis showed negligible radioactivity on elution.

It is clear therefore that the method of ionophoresis on paper can yield nucleotides free from all traces of contaminating inorganic phosphate which might distort activity measurements. The method was therefore applied to the nucleotide fraction obtained by the Schmidt-Thannhauser (1945) method as described in procedure 1 above, using the liver tissue of rats which had received radioactive inorganic phosphate by subcutaneous injection 2 or 24 hr. before killing.

The result of ionophoresis as shown in an ultraviolet light print is given in Fig. 1 which reveals that the four nucleotide spots are preceded by a fast-moving crescentic spot which absorbs ultraviolet light faintly.

To demonstrate the presence of this unknown component which we have called *A*, a short run on a 57 cm. paper strip at 14 V./cm. for 6 hr. sufficed, but this was inadequate for the complete separation of the four nucleotides. In the normal procedure for good nucleotide separation on a longer paper the fast-moving components run off the end. For each solution two runs were therefore usually employed—a short one for the separation of the fast-moving component (Fig. 1) and a conventional longer run on a 72 cm. paper for nucleotide separation (Fig. 2). The fast-moving component *A* was present both in solutions acidified with perchloric acid and in solutions prepared by acidification of the alkaline digest with TCA and subsequent removal of TCA with ether. It is discussed in greater detail below.

An ultraviolet photograph and an autoradiograph from a short run are shown side by side in Fig. 1. The four nucleotides are not adequately separated in the ultraviolet photograph, although they appear as discrete spots in the autoradiograph. The characteristically crescent-shaped spot *A* is clearly visible in both photograph and autoradiograph, but is more pronounced in the latter. Between *A* and uridylic acid lies a spot *C*, clearly visible in the autoradiograph, while uridylic acid itself is accompanied by two more spots *D* and *E* lying immediately in front of and behind it respectively. An additional spot *F* which appears in the autoradiograph lies behind cytidylic acid.

It is clear, therefore, that the RNA fraction obtained in the Schmidt-Thannhauser separation contains, in addition to the expected four nucleotides, at least five other compounds containing phosphorus, one of which can be shown up by ultraviolet absorption.

The results of a long ionophoresis run are shown in Fig. 2 in which components *A* and *C* have passed beyond the end of the paper. Here the nucleotide separation is adequate, but uridylic acid is still accompanied by components *D* and *E*, which show up on the autoradiograph but not in the ultraviolet photograph. These two components remain very close to uridylic acid and do not separate as discrete spots. Since their specific activity is much higher than that of uridylic acid, the effect of their presence is to make uridylic acid appear unduly active.

strips. When the ultraviolet-absorbing area *A* was eluted it was found possible to separate the eluate into an inorganic and organic constituent, both of which contained phosphorus. The inorganic phosphate content was determined by adding sulphuric acid, ammonium molybdate and 2:4-diaminophenol hydrochloride to a portion of the eluate without digestion. The intensity of the blue colour was measured giving the concentration of inorganic phosphorus, and the blue phosphomolybdate complex was then extracted with *iso*butanol for radioactive assay. Radioactive measurements were also carried out on the aqueous layer. A second portion of the eluate was used for determination of total phosphorus. Organic phosphorus was determined by difference.

The inorganic portion was designated *B* while the organic portion, which must be responsible for the

Table 1. *Separation by ionophoresis of the components of the ribonucleotide fraction of rat-liver tissue submitted to the modified Schmidt & Thannhauser method (procedure* 1)

(For an explanation of *A*, *B*, *C*, *D* and *F*, see Fig. 1 and text. Results are expressed in μg. P/100 μg. P in the whole fraction.)

Exp. no.	Adenylic acid	Guanylic acid	Cytidylic acid	Uridylic acid	*A*	*B*	*C*	*D*	*F*	Recovery (%)
1	14·2	24·2	20·6	16·7	4·3	3·8	6·1	6·5	—	89·1
2	14·6	25·0	24·0	16·4	1·9	2·2	8·1	8·0	—	96·2
3	13·3	23·5	20·8	15·3	1·4	1·5	9·7	11·0	—	96·5
4	13·2	25·4	21·5	15·9	3·5	2·0	6·9	3·0	—	91·2
5	14·0	26·4	20·6	15·9	3·8	2·5	3·4	6·2	—	92·8
6	12·8	25·8	19·9	18·2	4·6	5·6	8·7	4·6	—	100·2
7*	12·3	28·4	19·7	15·4	6·0	1·5	4·5	4·2	1·1	93·1

* Liver cytoplasm only.

The relative amounts of phosphorus in the component parts of the ribonucleotide fraction obtained in procedure 1 above (the A_2S fraction in the modified Schmidt & Thannhauser procedure used by Davidson *et al.* 1951) are shown in Table 1. The figures for nucleotides were obtained from long ionophoretic runs and those for the concomitants *A*–*F* by the corresponding short runs. The total recovery of phosphorus from all components is usually less than 100%, but it is clear that the nucleotides together comprise some 75% of the total phosphorus in the fraction. The relative amounts of the nucleotides are of the same order as those obtained from isolated liver RNA (see previous paper). The amounts of each concomitant vary quite widely from experiment to experiment. This may be due in part to the arbitrary way in which the paper was cut, since considerations of time made it impossible to prepare an autograph from each strip before it was cut up, eluted and counted, and in part to the fact that the amount of phosphorus in each eluate was at the lower limit of accuracy of the method of estimation. This precluded the use of more numerous but narrower ultraviolet absorption, retained the name *A*. The presence of the inorganic component *B* accounts for the fact that the width of the band on the autoradiograph is considerably greater than that on the ultraviolet photograph. The other components *C*, *D* and *F* contained no inorganic phosphate, nor were they visible on examination of the paper in ultraviolet light. The relative positions occupied by all these components and by the nucleotides on the paper is apparent from the photographs in Fig. 1.

The specific activities of the various components listed in Table 1 are shown in Table 2. It will be seen that the activity of the whole fraction is much higher than that of any of the nucleotides. This is due to the high activity of the concomitants and particularly of the inorganic component *B* which corresponds to the 'phosphoprotein' phosphorus or P_2 fraction of Davidson *et al.* (1951) which likewise had a very high activity. The discrepancy between the activities of the nucleotides and of the whole fraction is most noticeable 2 hr. after injection. After 24 hr. the activity of the nucleotides has increased while that of the concomitants has decreased.

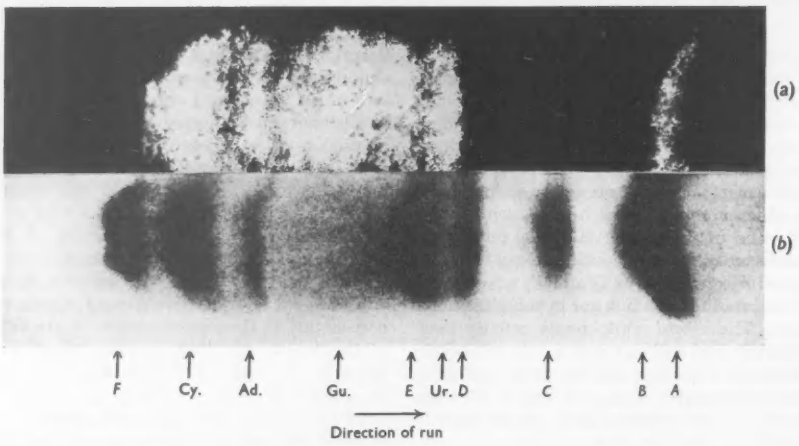

Fig. 1.

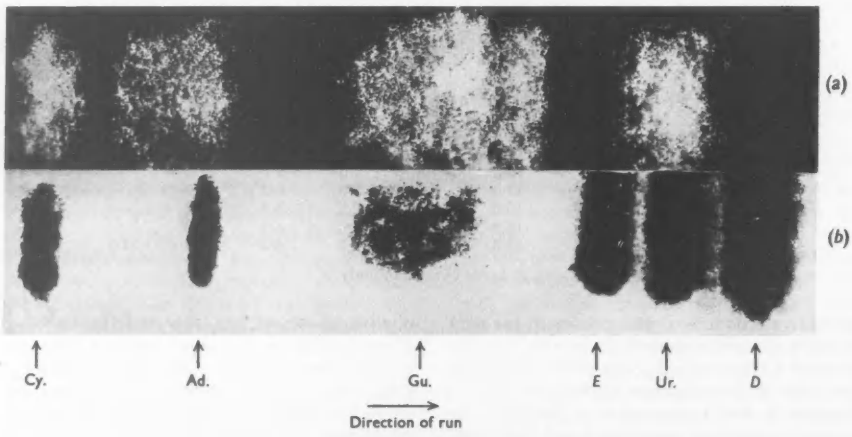

Fig. 2.

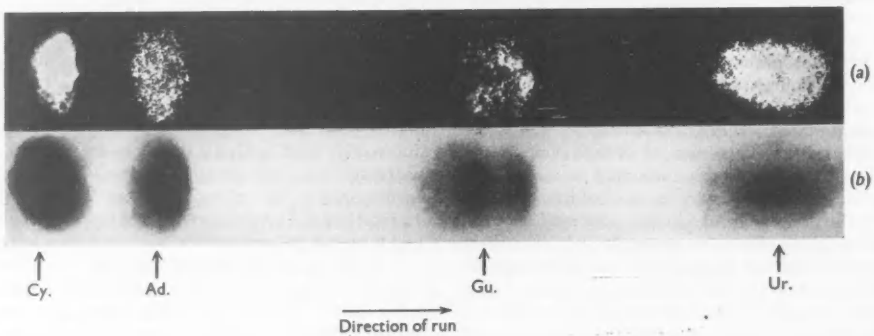

Fig. 3.

Of the three nucleotides adenylic acid, guanylic acid and cytidylic acid, the first was generally found to have the highest activity although the differences between them were not large. Uridylic acid, on the other hand, showed an activity so much higher than any of the other nucleotides that it was clear that contamination by components *D* and *E* was occurring. Accordingly, the areas corresponding to *D* and *E* were cut out and eluted along with the uridylic acid itself as obtained from a long run. Although uridylic acid showed a higher activity than the other three nucleotides, the concomitants *D* and *E* showed still higher values (Table 3). It would appear probable therefore that values obtained for uridylic acid in ionophoretic runs of this fraction would be liable to exhibit excessively high activities owing to the likelihood of contamination by closely associated highly active concomitants. The presence of *D* and *E* is clearly shown in the autoradiograph in Fig. 2.

It was thought that the presence of concomitants *A–F* might be due in part at least to incomplete removal of acid-soluble or lipid phosphorus during the preliminary extraction procedure. Experiments were therefore carried out in which the number of extractions with 10 % TCA was increased to five and the lipid extraction was supplemented by two extractions of 30 min. with a boiling ethanol-chloroform (3:1) mixture. Subsequent analysis revealed that neither procedure had reduced the concomitants in number or amount. Of these concomitants the most troublesome are *D* and *E* which lie in close

Table 2. *Specific activities of the phosphorus compounds separated by ionophoresis of the ribonucleotide fraction obtained by the modified Schmidt & Thannhauser method (procedure* 1)

(The results were obtained from the livers of rats which had received 50 μc. ^{32}P/100 g. body weight at different intervals before killing. For component *E* see Table 3.)

Time after injection (hr.)	Specific activity (counts/min./100 μg. P)									
	Adenylic acid	Guanylic acid	Cytidylic acid	Uridylic acid	*A*	*B*	*C*	*D*	*F*	Whole fraction
2	485	184	344	660	5075		1350	1590	—	—
2	723	431	490	990	1500	7800	2000	2600	—	2050
2	715	390	565	920	500	19800	2350	2800	—	1780
2	975	570	810	870	4160	21300	6700	2300	—	2560
2	293	278	275	702	1820	3120	1180	775	—	1280
2	300	255	215	588	2470	4270	1120	3020	—	1190
2	287	249	234	525	2000	3320	1000	1720	—	1050
2	1240	785	1165	2325	487	60500	3960	3400	6750	3500
24	2150	2090	2040	2290	950	2550	3600	2650	—	2850
24	2000	1960	1840	2340	5700		4180	3050	—	2780

Table 3. *Specific activities of the four nucleotides and of the eluates from D and E (Fig.* 2), *obtained in a long ionophoresis run of the ribonucleotide fraction obtained by the modified Schmidt & Thannhauser method (procedure* 1)

(Rat liver, 50 μc. ^{32}P/100 g. body weight administered 2 hr. before killing.)

Exp. no.	Specific activity (counts/min./100 μg. P)						
	Adenylic acid	Guanylic acid	Cytidylic acid	Uridylic acid	*D*	*E*	Whole fraction
1	559	323	469	749	2660	1595	2360
2	978	434	838	1110	2805	2030	4280

Ultraviolet prints (above) and autoradiographs (below) of the corresponding ionophoretic runs of the nucleotide fractions of liver tissue. Cy., cytidylic acid; Ad., adenylic acid; Gu., guanylic acid; Ur., uridylic acid.

Fig. 1. Short ionophoretic run of the nucleotide fraction prepared by procedure 1. Components *A–F* are visible in the autoradiograph, but only *A* shows up in the ultraviolet print. The nucleotides have not yet separated from each other.

Fig. 2. Long ionophoretic run of the ribonucleotide fraction prepared by procedure 1. Separation of the nucleotides is adequate, but uridylic acid is accompanied by components *D* and *E* which appear in the autograph but not in the ultraviolet print.

Fig. 3. Long ionophoretic run of the ribonucleotide fraction prepared by procedure 2. The nucleotides are adequately separated and no concomitants are visible. The tendency for the isomeric guanylic acids to separate is seen on the autoradiograph.

association with uridylic acid. Attempts to separate them by extending the length of the run and altering the pH were unsuccessful.

In several cases, ionophoresis of a radioactive ribonucleotide fraction (procedure 1) was carried out down one edge of a wide strip of paper, the paper dried in the usual fashion, and then subjected to chromatography in the form of an ascending chromatogram at right angles to the direction of ionophoresis. The solvents used were the *iso*butyric acid/ammonia, *iso*propanol/hydrochloric acid and methanol/formic acid systems of Magasanik, Vischer, Doniger, Elson & Chargaff (1950), Wyatt (1951) and Bandurski & Axelrod (1951), respectively. The uridylic acid spot obtained in this way showed a reduced activity comparable with that of the other nucleotides, but this combined ionophoresis and chromatography was cumbersome, and at the best semi-quantitative.

Table 4. *Specific activities of the nucleotides separated by ionophoresis of the nucleotide fractions, obtained by procedure* 1 (*modified Schmidt & Thannhauser method*), *and procedure* 2 (*partial isolation method*)

(Rat liver, 50 μc. ^{32}P/100 g. body weight administered 2 hr. before killing.)

	Specific activity (counts/min./100 μg. P)	
Nucleotide	Procedure 1	Procedure 2
Adenylic acid	482	565
Guanylic acid	412	370
Cytidylic acid	430	455
Uridylic acid	1335	562
Whole fraction	1355	540

Accordingly, an attempt was made to obtain nucleotide material free from the concomitants by carrying out a partial isolation of RNA by sodium chloride extraction as described in procedure 2 above. The results of such an experiment are shown in Table 4 in which the specific activities of the nucleotides obtained by ionophoresis of the ribonucleotide fraction from a Schmidt & Thannhauser separation (procedure 1) are compared with those obtained by ionophoresis of the material extracted in procedure 2. An ultraviolet print and an autograph are shown in Fig. 3. Only the nucleotides and no concomitants are visible.

It is clear that procedure 2 yields a fraction containing no large proportion of highly radioactive contaminants since the specific activities of the nucleotides and of the whole fraction are comparable. Procedure 2, moreover, yields uridylic acid with an activity much lower than that found in procedure 1 and comparable with that found for the other nucleotides. The activities of the other three nucleotides obtained by the two methods are of the same order.

DISCUSSION

A useful application of the method of ionophoresis lies in the separation of the components of the ribonucleotide fraction of a tissue from an animal which has received a tracer dose of radioactive phosphorus. Davidson *et al.* (1951) have shown that when liver tissue from such an animal is submitted to the conventional separation procedure of Schmidt & Thannhauser (1945), the specific activity of the phosphorus in the ribonucleotide fraction (A_2S) is much greater than that in the RNA isolated from the same tissue. This difference could in part be accounted for by the presence in the ribonucleotide fraction of a small amount of highly active inorganic phosphate derived from 'phosphoprotein', but even after its removal, the activity of the residue (fraction S_2 of Davidson *et al.* (1951)) is still greater than can be explained by the presence of ribonucleotides alone. Similar drawbacks to the use of the Schmidt-Thannhauser procedure in experiments with ^{32}P have also been recorded by Marshak & Vogel (1950), by Jeener & Szarfarz (1950), and by Szarfarz & Paternotte (1951), who used paper chromatography in an attempt to free the ribonucleotides from radioactive contaminants such as inorganic phosphate.

By the use of the ionophoresis procedure to separate the components of the ribonucleotide fraction these difficulties can be resolved. The experiments with ^{32}P added to nucleotide mixture make it clear that ionophoresis can remove all traces of contaminating inorganic phosphate from the nucleotides. Such purification is virtually impossible by chemical methods alone, and ionophoresis therefore provides a satisfactory solution to the problem of determining true specific activities of the individual nucleotides.

The method of ionophoresis used in conjunction with autoradiography has revealed that of the ribonucleotide fraction obtained in the conventional Schmidt & Thannhauser separation, only some 75 % of the phosphorus is in fact derived from the ribonucleotides. The remaining 25 % consists of at least six protein-bound phosphorus derivatives which are released as acid-soluble compounds on incubation with alkali. Of these six, five are organic phosphates of unknown composition while the sixth (component *B*) is inorganic phosphate presumably derived from 'phosphoprotein' and corresponding to the fraction P_2 of Davidson *et al.* (1951). These authors pointed out that on the basis of pentose estimations nucleotide phosphorus accounted for not more than 85 % of the total phosphorus in the ribonucleotide fraction obtained by the Schmidt & Thannhauser method. Of the remainder only a small proportion could be determined as inorganic phosphate, and it is now clear that this is due to the

presence of the other concomitants. The Schmidt & Thannhauser (1945) method, as usually employed, tends therefore to give unduly high results for the RNA concentration in a tissue.

The chemical nature of the concomitants is at present under investigation. Only one of them (component *A*) exhibits any appreciable absorption in ultraviolet light. It has the unusual property of migrating as rapidly as inorganic phosphate. All the concomitants are present in all three cytoplasmic fractions corresponding to mitochondria, microsomes and cell sap (Smellie & Davidson, 1952). Similar concomitants, not necessarily identical with those found in the cell cytoplasm, are found in the cell nucleus (McIndoe & Davidson, 1952).

It is quite clear from our results that the nucleotide fraction obtained by the Schmidt & Thannhauser (1945) procedure is in itself useless as an indication of the specific activities of the nucleotides which it contains as has already been pointed out by Davidson *et al.* (1951). Separation of its component parts by ionophoresis yields reliable values for the specific activities of adenylic, cytidylic and guanylic acids. Uridylic acid, on the other hand, is so closely accompanied by components *D* and *E* that reliable specific activity figures cannot be obtained, and this accounts for the high activities for uridylic acid recorded by Davidson, McIndoe & Smellie (1951) and Davidson (1951). Moreover, the presence of these concomitants leads to high values for the phosphorus content of the uridylic acid fraction (Table 1).

The most satisfactory solution to the problem of obtaining the true specific activities of the nucleotides is to carry out a partial isolation of the RNA by our procedure 2, and to submit the material so obtained to ionophoresis.

This method yields material free from all concomitants including *D* and *E* which, especially the latter, cling so obstinately to uridylic acid. It is moreover much simpler and quicker than the exhaustive purification previously described (Davidson *et al.* 1951), but it should be noted that even this material contains traces of component *C* as does highly purified RNA.

The extraction of nucleic acid in procedure 2 is not quantitative, yields of about 70 % being usual. The material obtained, however, is satisfactory for determining the relative molar proportions of bases or the specific activities of the nucleotides although not for estimating the RNA content of a sample of tissue. The chief danger in its use is the possible preferential extraction of one RNA from material not homogeneous with respect to nucleic acid content.

Procedure 2, then, gives material which on ionophoresis provides a true measure of the specific activities of all four nucleotides (Table 4). Only by such separation of the component nucleotides can the true specific activity of an RNA be determined. The differences between the nucleotides are not great, but adenylic acid is in general the most active. Volkin & Carter (1951), who carried out similar experiments involving the separation of the constituent ribonucleotides of liver RNA by ion-exchange chromatography after the administration of much larger doses of ^{32}P 20 min. before killing, found the highest activity in adenylic acid and the lowest in guanylic acid.

SUMMARY

1. In experiments with ^{32}P the use of the procedure of ionophoresis on paper makes it possible to obtain nucleotides free from all traces of contaminating inorganic phosphate.

2. When the ribonucleotide fraction obtained by the procedure of Schmidt & Thannhauser (1945) from the tissues of an animal receiving ^{32}P is submitted to ionophoresis, at least six non-nucleotide phosphate derivatives can be detected as well as the four ribonucleotides. Their presence can conveniently be demonstrated by making autoradiographs of the ionophoretic run.

3. One of these six concomitants is inorganic phosphate derived from phosphoprotein. The other five are organic phosphates.

4. Two hours after administration of ^{32}P to rats and rabbits the concomitants all have higher specific activities than the nucleotides. This accounts for the abnormally high specific activity of the Schmidt-Thannhauser fraction.

5. A modified Schmidt-Thannhauser procedure involving partial isolation of the ribonucleic acid is described which, in combination with ionophoresis, gives a true picture of the specific activities of the ribonucleotides.

6. Ribonucleotides comprise only about 75 % of the total phosphorus in the ribonucleotide fraction obtained from tissues by the Schmidt-Thannhauser procedure.

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Studies on the Synthesis of Lactose by the Mammary Gland

2. THE SUGAR-PHOSPHATE ESTERS OF MILK

By MARY G. McGEOWN and F. H. MALPRESS
Department of Biochemistry, Queen's University, Belfast

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In the course of investigations on the intermediary metabolism involved in the synthesis of lactose in the mammary gland, we have made repeated attempts to detect phosphate esters of galactose and lactose, both in extracts of actively secreting guinea pig glands and in media containing similar tissue metabolizing glucose *in vitro* (Malpress & Morrison, unpublished; Malpress, 1950). The results have been disappointing. In isolated cases faint positive tests were obtained for both these sugars in chromatographic studies after acid hydrolysis or after the action of alkaline phosphatase preparations on solutions obtained by barium fractionation of the initial extracts, but the indications were too weak and inconsistent to allow any conclusion regarding the normal occurrence of such esters in the active gland synthesizing lactose.

It seemed possible, however, in view of reports by Graham & Kay (1933) and by others of the presence of acid-soluble phosphate esters in milk, that an analysis of milk by a barium fractionation procedure might provide a new approach and one that might well yield results of interest in connexion with the problems of lactose synthesis. This was the more likely, since theories of milk secretion (Richardson, 1947) recognize both the diffusion and the extrusion of milk constituents from the alveolar cells, and in the latter case the rupture or 'decapitation' of the cells might be expected to lead to the presence of intermediates of mammary cell metabolism in the milk.

This paper reports the results of such an investigation in which minute amounts of α-galactose-1-phosphate, glucose-1-phosphate, a second glucose phosphate (probably glucose-6-phosphate) and a lactosephosphate have been detected in normal milk; phosphopyruvic acid has also been found.

Hitherto, apart from the production of α-galactose-1-phosphate by lactose-fermenting or galactose-adapted yeasts (Trucco, Caputto, Leloir & Mittelman, 1948; Wilkinson, 1949), the only known formation of galactose esters in living organisms is that, again of galactose-1-phosphate, reported by Kosterlitz (1943) in the livers of rats fed on galactose, though Laszt & Sullmann (1935) and Verzár & Sullmann (1937) have adduced circumstantial evidence for the formation of these compounds in the intestine during the absorption of galactose. The esterification of galactose in the carbon-1 position depends, in yeasts, upon the presence of a galactokinase, and the work of Bacila (Trucco *et al.* 1948) suggests that the same mechanism operates in the liver.

We know of no reported occurrence of any lactose phosphate in animal tissue.

EXPERIMENTAL

Milk sampling. Six bulk samples of milk, varying from 8 to 16 l. in volume, were taken from tuberculin-tested herds and collected in a sterilized churn after passing from the cooler. Trichloroacetic acid (TCA) to 5% (w/v) concentration was added either immediately or on arrival at the laboratory, the time interval from milking in the latter case being about 30 min.

Fractionation. Two main procedures were adopted, the first designed to give a final solution for analysis containing all the sugar phosphates present in the milk sample, the second to contain only those which were stable to 0·1 N-HCl hydrolysis for 7 min. at 100°.

In the first case the clear, yellow TCA supernatant obtained after centrifuging the precipitated proteins and filtering off the fat layer was adjusted to pH 8·2 and the insoluble material removed. Excess barium acetate was then added and the solution treated with an equal volume of ethanol. The pH was readjusted to 8·2 and after standing overnight at 4°, the precipitate was centrifuged, washed twice with ethanol and twice with ether, dried in air and then dissolved as far as possible by repeated extraction with 0·01 N-HCl. The solution was then precipitated a second time with an equal volume of ethanol at pH 8·2. This second precipitate was washed and dried as before, dissolved in dilute acid and the barium removed by addition of sat. Na_2SO_4 solution; the barium-free solution was then extracted twice with equal volumes of ether to remove traces of contaminating lipid material. After removing any excess sulphate ion from the solution by addition of barium acetate, the ethanol-insoluble barium salts were precipitated a third and fourth time as before, the volumes from which each precipitate was obtained being approx. 18 l., 2 l., 500 ml. and 500 ml., respectively, for an initial milk sample of 12 l. At each stage, extraction of the barium precipitate with 0·01 N-HCl left a residue which was not readily soluble; provided, however, that the precipitate had been repeatedly extracted, these residues showed no evidence of sugar-phosphates on examination and were discarded. The barium-free solutions of the fourth precipitates were the starting-points for the analyses carried out on these samples.

In the second procedure the milk samples were treated by the method already outlined, with the following modifications. The barium-free solution from the third barium-ethanol precipitate was hydrolysed in 0·1 N-HCl for 7 min. at 100° to destroy the acid-labile galactose-1-phosphate and glucose-1-phosphate present. Since the solutions showed marked buffering ability, the acid strength was judged by indicator reaction using thymol blue; because the pH of the solutions increased during the hydrolysis, all final solutions were examined for traces of acid-labile phosphates which had escaped hydrolysis. In no case were such traces found. After hydrolysis, liberated phosphate and excess sulphate were removed as barium salts and the solutions precipitated twice more with excess barium acetate and an equal volume of ethanol at pH 8·2. The barium-free solutions of the final precipitates and of the barium hydroxide-soluble ethanol-soluble supernatants from the 0·1 N-HCl hydrolyses were used for analysis.

Polarimetry. Polarimetric determinations were made on barium-free solutions, after drying *in vacuo* over P_2O_5 followed by solution in 0·1–0·2 ml. 0·25 N-HCl; monochromatic light from a sodium lamp was passed through the solutions held in microtubes, length 0·5 dm., volume approx. 50 cu.mm. After obtaining the rotation values, the solutions were washed out of the polarimeter tube, diluted to a suitable volume, usually 10 ml., and samples taken for further analysis.

Chromatography. Sugars were identified chromatographically, using the general experimental technique of Partridge (1948). Salts were removed from the original solutions by the method of Malpress & Morrison (1949), with the following modifications: the solutions were invariably dried over P_2O_5 *in vacuo*, and the pyridine extractions were carried out at a temperature not exceeding 30°. The final solutions, as prepared for application to the paper, had a volume of 0·1 ml. or less. The solvents used were collidine, prepared from commercial products according to the directions of Partridge (1948), and *iso*butyric acid; colour development was by the methods of Trevelyan, Procter & Harrison (1950) or Bryson & Mitchell (1951). The R_G values given in this paper represent the ratio R_F (spot): R_F (glucose).

Alkaline phosphatase. This was prepared by the method of Schmidt & Thannhauser (1943), incubation with phosphatase being carried out at pH 9, in the absence of added buffer. Inorganic phosphate was estimated by the method of Fiske & Subbarow (1925), pyruvic acid according to Friedemann & Haugen (1943) and reduction by the iodimetric method of Somogyi (1945) using a boiling time of 30 min.

Fermentation. Solutions were incubated at 37° with shaking, in the presence of washed suspensions of baker's yeast. It was found that fermentation of standard galactose solutions always resulted in a slight loss of the hexose measured by reduction; this loss did not change with the length of the fermentation period and was probably due to the fact that the yeast cells could conveniently be washed only once after centrifuging them from the fermented solutions. The use of standard solutions, containing amounts of galactose comparable with those found in the experimental solutions, afforded correction values which were applied in the calculation of galactose concentrations; the corrections automatically took into account the slight positive 'blank' reducing value of supernatants from the yeast suspensions.

RESULTS

Analysis of barium-free solutions not subjected to acid hydrolysis during the fractionation procedure

Acid hydrolysis. Typical results for the increase in reduction values and in inorganic phosphate after hydrolysis for varying periods in 0·1 N-hydrochloric acid are shown in Fig. 1. In both cases (curves *A* and *D*) the quantities increased rapidly during the first 3 min. after which practically no further increase occurred. Only a small part of the liberated sugar was fermentable, and it was evident that the non-fermentable fraction had been completely liberated in 2 min. (curve *B*); curve *C* gives the increase in reduction after fermentation, corrected for galactose loss (see Experimental section). Hydrolysis in N-hydrochloric acid for 7 min. gave only slight increases in reduction or free phosphorus values over those given by the milder treatment.

Fig. 1 shows further that the original unhydrolysed solution contained both reducing substance and free phosphate. Hydrolysis with 0·25N-sodium hydroxide resulted in the disappearance of this initial reducing power, with a corresponding

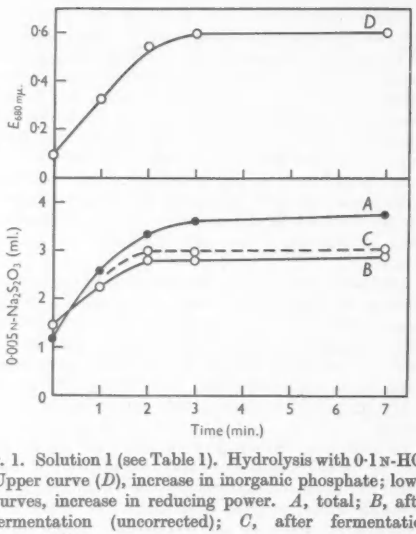

Fig. 1. Solution 1 (see Table 1). Hydrolysis with 0·1N-HCl. Upper curve (*D*), increase in inorganic phosphate; lower curves, increase in reducing power. *A*, total; *B*, after fermentation (uncorrected); *C*, after fermentation (corrected).

increase in the inorganic phosphate value; subsequent hydrolysis of the solution with 0·1N-hydrochloric acid showed that there had been no destruction of the acid-labile phosphates by this alkaline treatment, the absolute increase in reducing power and liberated phosphate being identical with that obtained in solutions not subjected to the alkaline hydrolysis.

Quantitative results from three solutions, based on the results of hydrolysis experiments and the chromatographic evidence reported below, are given in Table 1.

Chromatography. Samples of the barium-free solutions were analysed chromatographically: (1) after incubation for 3 hr. at 37° with alkaline phosphatase; (2) after hydrolysis for 3 min. with 0·1N-hydrochloric acid at 100°; (3) after hydrolysis for 7 min. with N-hydrochloric acid at 100° and (4) after hydrolysis with 0·25N-sodium hydroxide for 30 min. at 100°. The first three treatments gave identical qualitative chromatograms: galactose (strong), glucose (weak) and a third diffuse spot as yet unidentified, which in collidine had an R_G value of approximately 1·20 (very weak). Fermentation experiments resulted in the disappearance of the glucose spot only. Unhydrolysed solution or the solution incubated with boiled phosphatase preparations gave blank chromatograms, as did the alkaline hydrolysis (4).

Polarimetry. Two barium-free solutions, for which the analyses are given in Table 2, were examined after first subjecting them to alkaline hydrolysis (0·2N-sodium hydroxide for 30 min. at 100°) to destroy any sugar phosphates with free reducing groups, followed by one further barium-ethanol precipitation of the remaining alkali-stable phosphates. The second of these solutions (*B*, Table 2) was of particular importance since both chromatographic studies and reduction values before and after fermentation showed that it contained a galactosephosphate as the only acid-labile constituent.

Table 1. *Analysis of bulk milk samples*

Milk sample	Glucose-1-phosphate (mg./l.)	Galactose-1-phosphate (mg./l.)	$\frac{\text{Acid-labile P (calc.)*}}{\text{Acid-labile P (found)}} \times 100$ (%)	$\frac{\text{Acid-labile P (found)}}{\text{Acid-labile P + alkali-labile P}} \times 100$ (%)
1	0·25	0·91	75·2	70·8
2	0·30	0·88	84·0	—
3	1·36	2·10	91·2	80·5

* Acid-labile P calculated from figures in the two preceding columns.

Table 2. *Analysis of samples used for polarimetry*

Milk sample	Galactose-1-phosphate (μg.)	Glucose-1-phosphate (μg.)	Galactose (μg.)	$\frac{\text{Acid-labile P (calc.)*}}{\text{Acid-labile P (found)}} \times 100$ (%)
A	53	153	—	75·5
B (before hydrolysis)	0	334	—	81·4
B (after hydrolysis)†	—	—	227	—

* Acid-labile P calculated from figures for glucose-1-phosphate and galactose-1-phosphate.
† 3 min. 0·1N-HCl at 100°.

Table 3. *Possible theoretical and observed values for optical rotations in solutions A and B*

(Theoretical rotations calculated from the analytical figures for glucose-1-phosphate and galactose-1-phosphate given for these solutions in Table 2, assuming in turn their presence in the four possible combinations of α- and β-forms.)

	Calculated rotations				
Glucose-1-phosphate	α	β	α	β	Observed
Galactose-1-phosphate	α	α	β	β	rotation
Solution *A*	+0·28	+0·23	+0·10	+0·05	+0·30
Solution *B*	+0·48 (galactose-1-phosphate present alone)		+0·09		+0·48
Solution *B* (after hydrolysis)	+0·18 (α- and β-galactose equilibrium mixture)				+0·15

In Table 3 the observed rotations of these solutions are compared with the theoretical values obtained by assuming, in turn, the four possible combinations of the α- and β-configurations of the two sugar-1-phosphates determined by analysis (Table 2). It is seen from this that the galactose-phosphate ester was present in the α-form, a conclusion fully supported by the decrease in rotation of the solution when the ester was converted into the free sugar by acid hydrolysis (Reithel, 1945). It seems probable that the glucose ester also had the α-configuration, though this can be claimed with rather less certainty. (See Cori, Colowick & Cori, 1937; Colowick, 1938; Kosterlitz, 1939; and Reithel, 1945, for rotation values of hexose-1-phosphates.)

From these results we conclude that the barium-free solutions used in these analyses contained at least four substances, in addition to some free orthophosphate: α-galactose-1-phosphate (Kosterlitz, 1939); α(?)-glucose-1-phosphate (see Kosterlitz & Ritchie, 1943, for hydrolysis rates of glucose-1-phosphate in 0·1N acid); one or more reducing sugar phosphates, destroyed by alkaline hydrolysis, which, by inference from the chromatographic evidence after phosphatase action, would probably prove to be esters of glucose or galactose; and finally traces of the unidentified compound having R_G 1·20 in our chromatograms run in collidine. It may be inferred from the last column of Table 1 that the two acid-labile hexose-1-phosphates were, together, present in greater concentration in our solutions than the alkali-labile phosphates.

Analysis of supernatants and of barium-free solutions obtained after the destruction of acid-labile esters

The supernatant solutions, when prepared for chromatography, corresponded to a larger volume of the original milk than the samples used in the acid hydrolysis experiments already described; consequently the evidence of free galactose, glucose and of the compound having $R_G = 1·20$ was even more definite on the chromatograms. An additional and unidentified substance, presumably present in milk as an acid-labile ester, was also detected and had an R_G value in collidine of approx. 1·78.

Samples of the barium-free solutions of the acid-stable esters gave blank chromatograms after 0·1N-hydrochloric acid hydrolysis for 7 min. at 100°, thus providing satisfactory evidence that the acid-labile galactose- and glucose-1-phosphates had been completely destroyed in their preparation. Hydrolysis with N-hydrochloric acid for 1 hr. at 100° resulted in an increase in the reducing power of the solutions, partly due to liberation of a fermentable sugar; the change was accompanied by an increase in free phosphate. Both these effects could be paralleled by the action of alkaline phosphatase.

Chromatography. Chromatograms of the solutions after N-hydrochloric acid hydrolysis showed glucose (strong) and galactose (weak) spots whether the hydrolysis was continued for 1 or for 12 hr. The action of alkaline phosphatase, however, resulted in a different chromatographic picture; in this case glucose was always present in amounts comparable with those found after acid hydrolysis, but galactose was invariably absent. On the other hand, it was evident that lactose had been liberated by the enzyme.

This evidence suggests that these solutions contained a glucosephosphate, not hydrolysed by 0·1N-hydrochloric acid and therefore distinct from the glucose-1-phosphate already found; this may prove to be glucose-6-phosphate although the results do not allow any certainty on this point. Further, the presence of a lactosephosphate in milk is indicated. The observation that galactose is present in the solutions after N-hydrochloric acid hydrolysis, when considered in relation to its absence from the solutions after phosphatase action, points to its derivation from the breakdown of lactosephosphate; this is made more likely by the failure to detect any lactose in the acid-hydrolysed solutions.

Further analytical results

The barium precipitates obtained by the methods outlined in this paper had dry weights of 5–10 g. Hydrolysis with 0·1N-hydrochloric acid for 7 min. at 100° reduced the barium-precipitable fraction to about one-sixth of this amount, of which the phosphate esters reported here formed only a very minute part (<1%).

Although no systematic examination of the main bulk of the precipitated material was carried out, it was evident from the strong pentose reaction (Mejbaum, 1939) given by the supernatants obtained after barium-ethanol precipitation of solutions hydrolysed with 0·1N-hydrochloric acid, that nucleotides were almost certainly present before the hydrolysis. This observation confirms the finding of Kay & Marshall (1928) (see also Mandel & Bieth, 1948).

Positive tests were also obtained for pyruvic acid after hydrolysing the solutions for 1 hr. in N-hydrochloric acid at 100°, or after alkaline phosphatase action. The rate of release of the free acid by N-hydrochloric acid corresponded closely to that given by Lohmann & Meyerhof (1934) for phosphopyruvic acid.

Results confirming those obtained with bulk milk samples were given by samples drawn from single udder quarters using the sterile techniques of dairy bacteriological practice. These samples were of interest in showing the presence of the sugar phosphates in milk samples which gave zero counts of viable bacteria per ml. of milk, when plated on 'Yeastrol' milk agar and incubated for 24 hr. at 37°.

DISCUSSION

The evidence given in this paper indicates the presence of four sugar-phosphate esters and one carbohydrate breakdown product in normal milk; these are α-galactose-1-phosphate, α(?)-glucose-1-phosphate, glucose-6(?)-phosphate, a lactosephosphate and phosphopyruvic acid. In addition at least two other substances, probably present as phosphate esters, remained unidentified; they have R_G values in collidine of 1·20 and 1·78.

The experimental data, although primarily qualitative, were able to give approximate quantitative assessments of the ester concentrations. Apart from galactose-1-phosphate and glucose-1-phosphate, where quantities of the order of 1 mg./l. were found, the concentrations of the other esters were usually less than 0·1 mg./l. Such low values might be expected in view of the alkaline phosphatase activity in milk. Although the optimum pH for the enzyme is about 9, Graham & Kay (1933) have demonstrated appreciable hydrolysis of glycerophosphate added to milk and also a slow decrease in the ester phosphate values of normal milk when it is kept for a few hours at room temperature or incubated at 38°. It is most likely, therefore, that destruction of phosphate esters is proceeding steadily in the udder during the time between milkings, and that for this reason their concentrations in different milk samples might show large variations (see Table 1).

Some doubt still attaches to the true nature of the ester here termed glucose-6(?)-phosphate. In any tissue other than the mammary gland there would be far less hazard in attributing phosphorylation to the carbon-6 position in any glucose ester hydrolysed only slowly in acid solution; in mammary tissue, however, the formation of galactose from glucose (French, Popják & Malpress, 1952), by a mechanism as yet undetermined, with its implied inversion of groups at carbon-4, suggests other possibilities which our experiments are not able to exclude; for instance, the alternative that the ester is glucose-4-phosphate (Dursch & Reithel, 1952).

The behaviour of the disaccharide phosphates has been little studied, but the lactose ester found here, which is destroyed by N-hydrochloric acid at 100° fairly rapidly, and yet is unaffected by treatment with 0·1N-hydrochloric acid at 100° for a short period, shows a resemblance to the lactose-1-phosphate synthesized by Reithel & Young (1952).

The main interest of these experiments is in the light they may eventually throw on the problem of lactose synthesis. As has already been argued elsewhere (Malpress, 1950) α-galactose-1-phosphate is *a priori* an unlikely metabolite in the synthesis of lactose into which the β-form of the hexose is eventually incorporated. However, its presence in milk together with glucose-1-phosphate may possibly be regarded as circumstantial evidence for the presence of the galactowaldenase system (Trucco *et al.* 1948; Caputto, Leloir, Cardini & Paladini, 1950) in the mammary gland. In this case α-galactose-1-phosphate might have been formed as a result of the equilibrium α-galactose-1-phosphate ⇌ α-glucose-1-phosphate in mammary tissue. An alternative explanation of its occurrence could be its formation from small traces of free galactose; Grant (1936) has shown that although galactose could not act as a precursor for lactose formation in *in vitro* mammary gland experiments, it could not be recovered quantitatively after incubation with the tissue, and was evidently being metabolized to some extent; the possible presence of a galactokinase in the mammary gland cannot therefore be ignored, and with it, the possibility of α-galactose-1-phosphate formation by the direct phosphoryla-

tion of the free sugar. Both these interpretations, however, give only a minor significance to the appearance of this ester in the milk.

The demonstration of a lactosephosphate on the other hand, does bring more certain evidence for the view, hitherto held with very slender experimental support, that phosphorylation is of fundamental importance in lactose synthesis. An isolated instance of the occurrence of such an ester in a trichloroacetic acid extract of cow-udder tissue has already been reported (Malpress, 1950), and it seems probable that this compound may prove to be the immediate precursor of lactose in the mammary gland.

SUMMARY

1. Barium fractionation procedures, supplemented by chromatographic analysis, have shown that α-galactose-1-phosphate, α(?)-glucose-1-phosphate, glucose-6(?)-phosphate, a lactosephosphate and phosphopyruvic acid are present in trace amounts in normal milk.

2. The possible significance of these findings in relation to lactose synthesis is discussed.

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The Determination of Cholesterol by the Liebermann-Burchard Reaction

By A. P. KENNY

Clinical Laboratories, The Victoria Infirmary of Glasgow

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The methods adopted for the determination of cholesterol in blood can be divided into two main groups. Those in the first group, in which the principal variation is in the manner of extraction, all conclude with the application of the Liebermann-Burchard reaction (Liebermann, 1885; Burchard, 1889) to the final extract and comparison of the colour developed against a standard cholesterol solution similarly treated. Included in this are the methods of Myers & Wardell (1918), Leiboff (1924) and Sheftel (1944). In these methods the specimen is absorbed on a suitable absorbent and the cholesterol is extracted by means of a continuous extractor. With the methods of Sackett (1925) and Bloor (1928) the blood is extracted directly in a given volume of mixed solvent. Attempts have also

been made to estimate cholesterol directly in acid chloroform in the methods of Sols (1947) and Zuckerman & Natelson (1948).

Into the second group can be gathered all the methods which entail precipitation of the extracted cholesterol as digitonide, which may either be weighed, as in the methods of Gardner & Gainsborough (1927), Boyd (1933) or Man & Gildea (1933), determined gasometrically as in that of Kirk, Page & Van Slyke (1934) or by oxidation as in the method of Okey (1928). The disadvantage of the weighing procedure lies in the relatively large volumes of blood required, and of the gasometric and oxidation procedures in that special equipment and technical skill are essential. Schoenheimer & Sperry (1934) succeeded in applying the Liebermann-Burchard reaction to cholesterol isolated as digitonide from 0·2 ml. of blood.

It has been found that techniques involving the Liebermann-Burchard reaction, like many of the older colorimetric methods, require a more specific set of conditions than was formerly recognized if they are to be adapted to a final absorptiometric determination. The object of this investigation has been to study the factors which influence colour development in chloroform solutions of cholesterol and its esters, with particular reference to the use of selective filters, the effect of time, temperature, exposure to light and the proportions of reagents, and to explain or eliminate the so-called spurious colours which so often develop and interfere with final evaluation. On the basis of these results it was hoped to evolve a method suitable for routine use in clinical laboratories and establish normal limits for such a method. A preliminary communication on part of this work has already been made (Kenny, 1948).

EXPERIMENTAL AND RESULTS

The instrument employed throughout this work was a Hilger Spekker absorptiometer, using spectral filters of the series H 558 and cells of 1 cm. thickness.

Effect of selective filters

The scheme adopted to determine the effect of selective filters upon the extinction value consisted of setting up a series of identical tubes, each containing 1 mg. of cholesterol in 10 ml. $CHCl_3$. The tubes were lightly stoppered and placed in the dark in a thermostatically controlled water bath at the temperature being investigated. After the contents of the tube had attained the temperature of the bath, 2 ml. acetic anhydride were added and the tubes shaken, then 0·2 ml. conc. H_2SO_4 was measured in, and the tubes finally inverted and returned to the water bath. At prearranged intervals, the tubes were removed from the bath, and the extinction values of the contents determined on the absorptiometer, a different pair of filters being used for each successive tube. Fig. 1 shows the absorption curve for the eight filters of the series, the colour having been developed at 20 and 40° for 10 and 30 min. in each case. The curves show two main maxima, one at about 430 mμ. and the other between 600 and 690 mμ.

Using a spectrophotometer, Morgareidge (1935) was able to demonstrate three absorption bands in the colour given in the Liebermann-Burchard reaction by cholesterol and certain related sterols, one at 450 mμ., a second at 620 mμ. and a third at 675 mμ. The 600–690 mμ. maximum shown in the absorptiometric measurements presumably represents the second and third peaks.

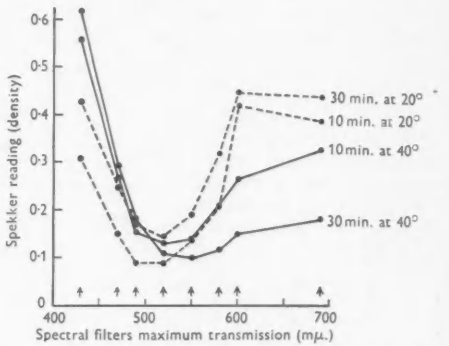

Fig. 1. Absorption curves for a solution of 1 mg. cholesterol in 10 ml. $CHCl_3$ + 2 ml. acetic anhydride + 0·2 ml. conc. H_2SO_4, for the eight Ilford filters of the series, H 558. Solutions were developed in the dark at 20 and 40° for 10 and 30 min. as indicated.

At 40° the development of colour was found to be more rapid than at 20°, and the absorption at 430 mμ. had considerably increased, while the 600 and 690 mμ. readings had fallen. There is, therefore, evidence of a shift in the intensity of the absorption bands. The explanation may be that the colour is due not to a single substance or, more probably, that this substance can be transformed from one form to another, since the loss in the blue-green coloration, absorbing at 690 mμ., is not paralleled by a loss in the yellow, absorbing at 430 mμ.

The findings would indicate that in an estimation the choice of filter lies between a red which estimates a thermolabile component of the colour or a blue which is not influenced by this component. Further data on this point are provided in the next section.

Effect of temperature

The importance of the control of temperature has been generally recognized, but the methods suggested by different investigators have shown considerable variation, although later workers have been more precise in this connexion. The following results show that such care is indeed warranted, especially with photometric measurements.

With the effect of filters in mind, a more detailed study of the influence of temperature was made. Series of tubes were set up as previously described and incubated at specified temperatures, one sample tube being removed at each time interval. The graded effect of temperature upon colour

development, using a filter with maximum transmission at 430 mμ., and also the same tubes read against a 690 mμ. filter is illustrated in Fig. 2.

These results have been found to confirm and extend the work of Hoffman (1940). It will be seen that adding the reagents and maintaining at 0° 'freezes' the system, and over a period of 30 min. only a trace of colour develops. Transferring the tube, even after 30 min., to a higher temperature, results in the development of a colour along, but slightly below, the curve obtained if the whole reaction were at the new temperature conditions.

ultimate value of the 430 mμ. reading when complete conversion to the yellow component had taken place, and appears to be a characteristic of the concentration. This point is further illustrated in Table 1 where readings are shown for both filters, the colour having been developed at 20 and 40° for periods up to 3 hr. The continuing rise in the readings with the 430 mμ. filter, and the corresponding fall in the 690 mμ. filter are clearly indicated. An analysis of the results plotted in Fig. 3 also supports this contention but reveals minor discrepancies attributable to deviation from Beer's law.

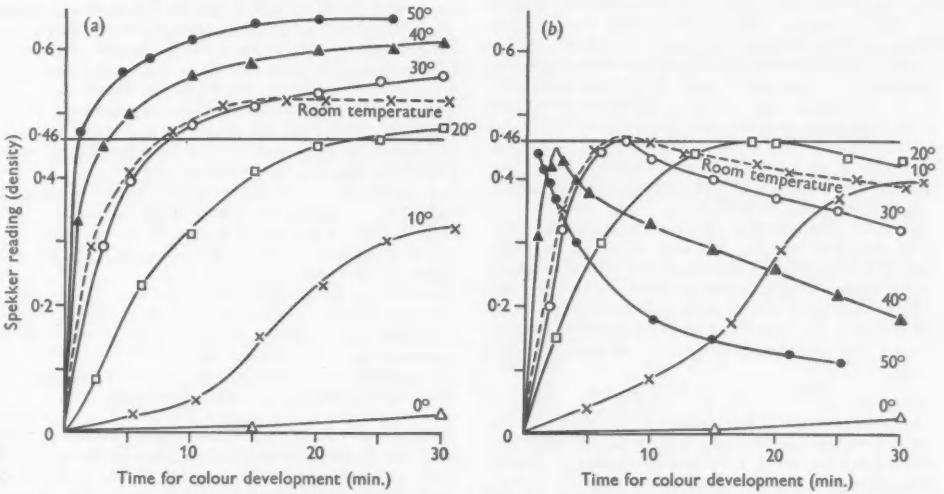

Fig. 2. Absorption curves for 1 mg. cholesterol in 10 ml. $CHCl_3$ + 2 ml. acetic anhydride + 0·2 ml. conc. H_2SO_4, developed at temperatures from 0 to 50° and plotted for a period of 30 min. with filters having maximum transmissions at (*a*) 430 mμ. and (*b*) 690 mμ. A 'room temperature' development curve is also shown.

The general effect of increasing the reaction temperature with the 430 mμ. filter was to give a more rapid colour development and a greater sensitivity. With the 690 mμ. filter, on the other hand, the maximum absorption was found to reach the same critical level for all temperatures of 20° and over. After this point the absorption at 690 mμ. diminished while, as stated above, that at 430 mμ. continued to increase.

The area enclosed between the temperature curve, the 0·46 abscissa and the 30 min. ordinate beyond the maximum point with filter 690 mμ., was found to be approximately 1·5 times the corresponding area when using the 430 mμ. filter. In the first case the extinction is on the decline and the area is below the 0·46 abscissa, whilst in the second, with the increasing extinction, it is above this line. This would indicate a constant conversion of the green to the yellow component, beyond the point where the extinction-time curve has reached its maximum with filter 690 mμ. The rate of development of the green component and of its conversion to the yellow is dependent principally upon temperature. As a consequence, any reading taken on a 430 mμ. temperature curve at or beyond 0·46 added to 0·67 of the reading on the corresponding 690 mμ. curve gives a constant reading of approximately 0·75, which would represent the

Variations in the concentration of cholesterol resulted in curves which all showed the same general character, but were set at higher or lower levels as the case may be. It is interesting to note that if colour be developed at room temperature, around 20°, it follows closely the 30° curve.

Table 1. *Effect of time on colour development*

(A continuation of the results plotted in Fig. 2.)

Temperature (°)	Maximum transmission of filter (mμ.)	Spekker readings			
		30 min.	1 hr.	2 hr.	3 hr.
20	430	0·48	0·53	0·56	0·58
	690	0·43	0·40	0·31	0·28
40	430	0·62	0·64	0·69	0·72
	690	0·18	0·12	0·07	0·05

When the 10 ml. of $CHCl_3$ solution are placed in a test tube in an open rack and treated with 2 ml. acetic anhydride, there is a rise in temperature of 4° and on the addition of 0·2 ml. conc. H_2SO_4 a further rise of 8° occurs, after which the normal cooling curve sets in and the contents of the

tube return to within 1° of room temperature in about 30 min.

From the results it would appear that 30 min. development at 37° would be a satisfactory arrangement when using a filter of maximum transmission at 430 mμ., since most laboratories possess an incubator or thermostat normally maintained at this temperature.

Reaction of esters

Cholesterol oleate and stearate were examined under the same conditions as outlined above and were found to give the same general pattern as did cholesterol. Several authors (Gardner & Williams, 1921; Reinhold, 1936; Yasuda, 1936; Kelsay, 1939) have reported that increased colour values are obtained from equivalent amounts of the esters as compared with the free sterol, and these enhancements have been reported as ranging from 5 to 30%. In the present experiments these esters were found to give a value 13% higher than cholesterol itself when calculated on an equivalent basis. This is a point which must be borne in mind when dealing with a mixture of free and esterified cholesterol such as exists in a Bloor extract.

The same maximum in the extinction-time curve was found with filter 690 mμ. but occurred at an extinction value of 0·52 in the case of the esters when estimated against a 1 mg. level. The rate of colour development over the range 0–40° was not significantly greater than for cholesterol itself and could not be taken as a basis for differentiating between free and combined cholesterol as in the method of Reinhold (1935).

Effect of time of development

The time recommended in certain of the earlier methods was 15 min. and, as seen from Fig. 2, could be applied with a fair degree of accuracy to a 'room temperature' development, or to temperatures close to 25°. With more precise temperature control, none of the curves reaches a maximum with the 430 mμ. filter even after 30 min., although the gradient of the curve is very small at this point with temperatures above 30°. With filter 690 mμ. the maximum depends upon the temperature, being 2·5 min. for 40° and 19 min. for 20°.

Table 1 shows readings obtained at intervals up to 3 hr. after the addition of the H_2SO_4, and represents an extension of the 20 and 40° results plotted in Fig. 2.

The effect of exposure to light

The deleterious effect of light upon the coloured product was also early recognized, but again the measures taken varied from developing in complete darkness to developing in the illumination existing in that part of the laboratory where the comparison was made.

The gross effect of light is demonstrated in the following experiment. Into three ordinary soft-glass tubes were pipetted 10 ml. $CHCl_3$ containing 1 mg. cholesterol. The tubes were placed in a thermostat and brought to a temperature of 40°. Acetic anhydride and H_2SO_4 were added as previously described, and when the temperature of the contents had fallen to 40°, two tubes were removed, one being clamped at an angle of 45° to horizontal above a 125 W. Osira H.P. mercury-vapour lamp, and the other 20 cm. above an ordinary 100 W. frosted filament lamp, in such a position as to maintain their temperatures exactly at 40°. The third tube was left in the thermostat (at 40°). All three tubes were allowed to develop for 30 min., after which the extinction value was measured.

A similar set of experiments was conducted using silica test tubes; the results are shown in Table 2. It is clear that only the yellow component absorbing at 430 mμ. is affected, and that under the conditions of the test the white light was more effective than the ultraviolet.

In order to demonstrate that this effect was not due to structural alteration within the cholesterol molecule because of irradiation, a similar series of tests was performed exposing the $CHCl_3$ solutions alone for 30 min., under the same conditions, then adding the other reagents and developing the colour in the dark thermostat for a further 30 min. The above assumption was shown to be correct since there was no appreciable difference between the readings of these cholesterol solutions and the control solution which had not been exposed. It therefore follows that the effect of white light is upon the yellow-coloured product itself, and although exaggerated in this experiment, it emphasizes the advisability of developing the reaction in the dark, or at least in a uniform subdued light.

Effect of proportions of reagents

Variations in the proportions of reagents materially influenced the final colour. With more than 0·3 ml. H_2SO_4 two layers separated and no measurement could be made. With

Table 2. *Effect of illumination upon colour development*

(Reagents: 2 ml. acetic anhydride followed by 0·2 ml. conc. H_2SO_4.)

		Spekker readings		
		Illumination		
General experimental conditions	Filter (mμ.)	Ultraviolet lamp	100 W. lamp	Dark thermostat
(*a*) 10 ml. $CHCl_3$ containing 1 mg. cholesterol in thin-walled soft-glass test tube. Reagents added and maintained at 40° for 30 min.	430	0·342	0·261	0·621
	690	0·174	0·170	0·178
(*b*) As above, but maintained first at 40° for 30 min., then reagents added and developed for a further 30 min. at 40° in dark thermostat	430	0·630	0·632	0·630
	690	0·177	0·175	0·170
(*c*) As in (*a*), but employing silica test tubes	430	0·452	0·395	0·620
	690	0·179	0·172	0·178
(*d*) As in (*b*), but employing silica test tubes	430	0·611	0·628	0·618
	690	0·180	0·168	0·178

0·3 and 0·2 ml. almost identical results were obtained, but 0·1 ml. and smaller amounts gave curves which were distinctly lower and the points of which showed a wide degree of scatter and were very variable. This latter effect may in part be due to difficulty in measuring such small volumes of concentrated acid.

The colour increased with increasing amounts of acetic anhydride up to 4 ml., but the dilution caused by the larger final volume almost compensated for the improved colour values.

Previous mixing of the acetic anhydride and H_2SO_4 gave almost identical sensitivity but a somewhat slower rate of colour development, which could be explained on the basis of the temporary increase in temperature following the exothermic reaction caused by the separate addition of these reagents.

Compliance with Beer's law

Using 2 ml. acetic anhydride followed by 0·2 ml. H_2SO_4, since these proportions only had been studied in detail, extinction values obtained with cholesterol up to 3·2 mg. in 10 ml. $CHCl_3$ showed an almost linear relationship with filter 430 mμ., and only slight deviation from linearity with filter 690 mμ., up to the limit of the instrument.

Fig. 3 shows calibration curves for 30 min. colour development at 20 and 40° with both filters and also for 5 hr. development at 40°. This latter result gave evidence of the stability of the yellow component and its suitability for colorimetric or absorptiometric estimation of cholesterol.

Differences between cholesterol concentration in serum and oxalated plasma

Schmidt (1935) first showed differences between the total cholesterol content of heparinized blood plasma and oxalated plasma. He found the former to give values which were 10–15% higher. Sperry & Schoenheimer (1935) found differences up to 15% between serum and plasma and showed that these differences affected the free and combined cholesterol to the same extent.

Because of the hypertonicity due to the added potassium oxalate, one would expect differences of the order of 2% between serum and oxalated plasma, since there is a shrinkage of about 3·5% in cell volume when 20 mg. potassium oxalate are added to 10 ml. of blood of normal red-cell content, according to Osgood (1926).

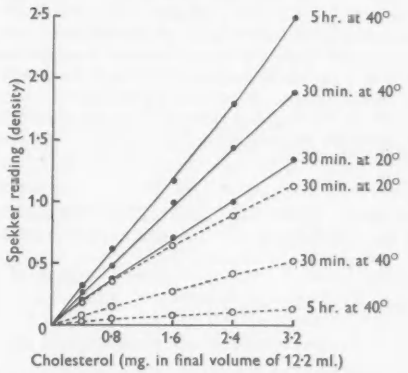

Fig. 3. Calibration curves for the estimation of cholesterol by the Liebermann-Burchard reaction. The cholesterol was dissolved in 10 ml. $CHCl_3$ and 2 ml. acetic anhydride added, followed by 0·2 ml. conc. H_2SO_4. Colours were developed at 20 and 40° for either 30 min. or 5 hr. as indicated. ——, filter 430 mμ.; - - - -, filter 690 mμ.

The results in Table 3, *a* and *b*, were obtained in experiments to compare the cholesterol content of serum and heparinized and oxalated plasmas by the method outlined in the next section. As seen from Table 3*a* the serum cholesterol remained constant for 24 hr., whilst the heparinized plasma was initially the same as the serum, but fell over the same period. The oxalated plasma showed an initial value 7% below that of the serum, but tended to rise somewhat over the next 24 hr.

Table 3. *Differences between total cholesterol levels in serum, heparinized and oxalated blood plasmas*

(Cholesterol expressed as mg./100 ml.)

(*a*)

Specimen	Time after withdrawal: 10 min. Packed cell vol. (%)	10 min. Cholesterol	1 hr. Cholesterol	4 hr. Cholesterol	24 hr. Packed cell vol. (%)	24 hr. Cholesterol
Serum	—	172	—	—	—	171
Heparinized plasma	45	173	168	—	45	164
Oxalated plasma	43	160	162	162	43	164

(*b*)

Case no.	Total cholesterol: Serum	Plasma	Serum/plasma	Case no.	Total cholesterol: Serum	Plasma	Serum/plasma
1	158	147	1·07	6	255	232	1·10
2	172	160	1·07	7	263	234	1·12
3	182	156	1·17	8	296	253	1·17
4	185	170	1·09	9	317	279	1·13
5	220	201	1·09	10	1260	1130	1·12
						Average	1·11

The ratio of serum to oxalated plasma cholesterol in the ten samples analysed, and shown in Table 3*b*, gave an average value of 1·11.

On the results of Sperry & Schoenheimer (1935), the differences cannot be attributed to hydrolysis of the esters by lipase, nor can it be explained as being due to absorption of cholesterol on the calcium oxalate precipitate. Since the heparinized sample was initially almost identical with the serum it cannot be related to clot formation. The explanation may lie in a surface phenomenon resulting in the absorption of cholesterol on the red cells as a consequence of the increased potassium ion concentration of the plasma, and investigations are proceeding in this direction to examine this possibility.

METHOD FOR THE DETERMINATION OF CHOLESTEROL IN PLASMA OR SERUM

On the basis of the experimental findings of the earlier sections, the following modification of the method of Bloor (1928) is recommended for the routine clinical estimation of cholesterol in plasma or serum.

Reagents

(1) Ethanol-ether mixture: 3 vol. 95% ethanol are mixed with 1 vol. ether.

(2) Anhydrous chloroform.

(3) Acetic anhydride (Analar).

(4) Sulphuric acid (Analar).

(5) Cholesterol standard.

(*a*) Stock solution: dissolve 0·547 g. anhydrous cholesterol in 100 ml. chloroform.

(*b*) Single working standard: prepare a 1 in 50 dilution of (*a*) in chloroform.

(10 ml. = 250 mg. total cholesterol/100 ml.)

Note on correction for esters. It has been shown in an earlier section that cholesteryl esters give a colour equivalent to 13% more in terms of cholesterol, than does the free sterol itself. If one accepts 28% as the mean value for the free cholesterol fraction (Peters & Man, 1943), then a total cholesterol of say 200 mg./100 ml. would give a reading equivalent to 219 mg. in terms of free cholesterol. An adjustment has been made for this in the preparation of the standard.

Basing a calibration on this assumption it can be shown that variations in the free cholesterol fraction between 20 and 40% of the total would not cause an error of greater than $\pm 2\%$ in the total so determined.

Method

About 15 ml. ethanol-ether mixture are measured into a 25 ml. stoppered volumetric flask, and 1 ml. of the sample is pipetted in, slowly and with constant stirring. The contents of the flask are then carefully brought to boiling point by immersing in a boiling-water bath for a few seconds at a time. The mixture is maintained at the boiling point of the solvent for about 2 min. and is then cooled thoroughly and diluted to 25 ml. with the mixed solvent.

The extract is filtered through a no. 4, 9 cm. Whatman filter paper, the funnel being covered by a watch glass to reduce evaporation. A 5 cm. length of capillary tubing, sealed at its upper end, is placed in a 6×1 in. boiling tube and 20 ml. of the filtrate are added and the contents of the tube are taken to dryness in a boiling-water bath.

The last traces of solvent and moisture are removed by heating the tube in a drying oven at 100° for a few minutes. The capillary is broken and the residue in the tube is extracted with 20 ml. chloroform, the walls of the tube being scraped with a glass rod to loosen the particles adhering to the sides and the extraction is completed by heating just to boiling point.

The chloroform extract is filtered hot through a no. 1, 11 cm. Whatman filter paper, with precautions as described above, and cooled. The residue which remains in the tube has been found to give no colour with the reagents. Into a 25 ml. glass-stoppered test tube are pipetted 10 ml. of the final chloroform filtrate and the tube and its contents are immersed in a thermostat at 37°, when 2 ml. acetic anhydride are added, mixed with the contents of the tube, and allowed to stand for 3 min. to attain the temperature of the bath. Then 0·2 ml. of conc. sulphuric acid is added from a microburette and the tube inverted and returned to the thermostat for 30 min., being protected from direct light.

As a blank, 7 ml. of the residual chloroform extract are treated with 1·4 ml. of acetic anhydride and incubated for 30 min. This blank has been found to give a fairly constant reading, even with distinctly pigmented plasmas, and is usually equivalent to about 12 mg. cholesterol/100 ml.

An interval timer is set going when the tubes are placed in the bath, and if a number of tests are to be made sufficient time is allowed between the additions of acid to enable all the tubes to be read at 30 ± 2 min. The extinction value is determined using a filter with a maximum transmission at 430 mμ. and interpolated on a calibration curve. It is suggested that a quantity control chart, such as is used for sodium by Wootton, King & Maclean Smith (1951) should be made in order to establish the reliability of the method.

Distribution curve for normal plasma total cholesterol

For speed of separation and economy in material, plasma cholesterol values have been determined in this laboratory by the above method for a number of years, and the distribution curve shown in Fig. 4 has been obtained.

The results have been drawn from 100 consecutive samples obtained from voluntary donors, and 100 samples from individual patients in this hospital, who, in the course of routine examination showed on an initial determination a basal metabolic rate

falling within the range of -7 to $+14\%$ of standard and were not known to suffer from other conditions associated with abnormal blood cholesterol values.

All the subjects were in the post-absorptive stage. The ages of the individuals, which contained equal numbers of men and women, ranged from 17 to 70 years. The irregularities in the curve must be attributed to the relative smallness of the sample. There is a slight skewness in the distribution which is not altogether unexpected, but for simplicity in calculation a Gaussian distribution has been assumed.

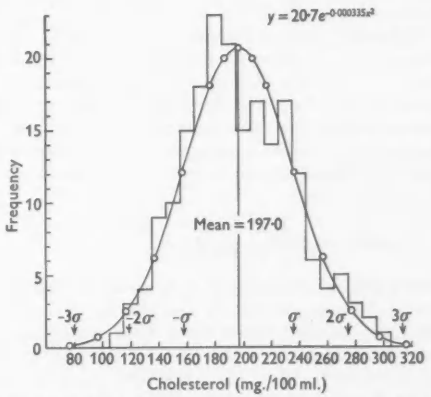

Fig. 4. Distribution curve for normal plasma total cholesterols. 100 males and 100 females, whose ages ranged from 17 to 70 years, are represented. The theoretical curve, $y = 20{\cdot}7e^{-0{\cdot}000335x^2}$, which assumes a Gaussian distribution, is indicated. Mean plasma total cholesterol, $197{\cdot}0 \pm 4{\cdot}1$ mg./100 ml.; standard deviation, 38·7 mg.

The mean value of the series was 197 mg./100 ml. and the standard deviation was 38·7 mg. It therefore follows that half the normal plasma cholesterols should fall within the limits 171–223 mg./100 ml., or $\pm 0{\cdot}67\sigma$, with a 1 in 20 chance of a normal falling outside the limits of 120–274 mg./100 ml., or $\pm 2\sigma$. Twenty replicate determinations on pooled normal plasma gave a result of $217 \pm 4{\cdot}3$ mg./100 ml.

DISCUSSION

A comparison of the recovery of cholesterol using various methods of extraction was made by Reinhold (1936), and he concluded that the method of Bloor (1928) gave complete extraction. Methods which involved drying of the sample followed by extraction generally gave lower recoveries. The work of Drekter, Bernhard & Leopold (1935) may give an explanation. They showed that cholesterol was present in two distinct fractions: one part was ether-soluble, and the other appeared to be in combination with protein and required denaturation by alcohol for its release. The method of Bloor has therefore been used throughout this work.

Gardner & Williams (1921) found that, of the two reagents employed in the development of the final colour, the quantity of sulphuric acid was the more important; considerable latitude could be allowed in the volume of acetic anhydride. Shapiro, Lerner & Posen (1935) suggested the use of a mixed reagent. As stated earlier, the main effect of this scheme of addition is to slow the initial stages of colour development, without greatly influencing the final result. Where small numbers of tests are being carried through, the mixed reagent holds no advantage, and may in fact be an inconvenience.

With the exception of Schube (1932) all authors appear to have recommended the measurement of the green colour, and where a filter is suggested it is one with a maximum transmission in the region of 650 mμ. Schube found that 15 min. development at 20° gave maximum colour for the green component, but he obtained more consistent results by making a second comparison between his standard and unknown after they had stood for 12–24 hr. in a dark cupboard, so that the yellow component was being measured.

The main disadvantage of measuring the yellow component, especially with a blue filter, would appear to be that any yellow pigmentation of the final chloroform extract would result in an increased blank. A correction can be applied by using part of the chloroform filtrate and adding only the acetic anhydride, but it has been found in practice that by far the greater part of any bile pigments present in a sample is absorbed on the protein precipitate during the extraction process. As an example, it was found in one instance where a plasma contained 17·3 mg. bilirubin/100 ml., that the blank, as described in the method, amounted to only 15 mg. cholesterol/100 ml.

The instability of the green colour has been the cause of much criticism being levelled at the Liebermann-Burchard reaction for the determination of cholesterol and has been responsible for the introduction of such artificial standards as naphthol green B by Reinhold (1935), and Carter's ink by Shapiro *et al.* (1935), and also certain schemes of washing such as that of Mueller (1916), in an attempt to eliminate the spurious yellows which are attributed to a fading standard. Sheftel (1944) was able to stabilize the green colour to some extent by the addition of glacial acetic acid.

The work of Hoffman (1940), as extended and discussed here, has clarified this position, and Sperry & Brand (1943), reviewing work on this reaction, stated that in their opinion the green colour was entirely empirical, no doubt implying that it was most variable, and that it was dependent

principally upon the factors of time and temperature, whereas the brown colour (described here as the yellow colour), appeared to be the end product and might be proportional to the amount of cholesterol. The present work has confirmed this view, because it has been shown that a relationship exists between the absorptions in the red and blue regions of the spectrum such that a constant can be obtained which is proportional to the amount of cholesterol present.

Sundeman & Razek (1937), using a Razek-Mulder colour analyser, showed that after 10 min. the reading at 530 mμ. alone remained constant, but the sensitivity was so low as to preclude its use for quantitative work. They found that in the 550–700 mμ. region the absorption increased with temperature, but was thermolabile. In the dark, increasing absorption took place in the blue-violet region, but the component measured at that wavelength was light-sensitive.

A decision has therefore to be taken on whether to measure the green component, making readings within a closely specified time, after developing at a temperature below 30°, or to measure the yellow component which is thermostable after a longer interval of time, and developed at a higher temperature in the dark, or in reasonable subdued light. The latter system has been adopted here.

Noyons (1938) found results 17% lower by the digitonide-precipitation method as compared with colorimetric determination, and Reinhold (1936) found differences averaging 10% in the same direction. These differences can be explained readily by the greater sensitivity shown by cholesteryl esters, and an allowance of 9% has been made for this in the preparation of the standard cholesterol solution.

Estimating normal serum cholesterols by their digitonide-precipitation method, Man & Gildea (1933) found a mean value of 208 mg./100 ml. with an experimental error of ±5·5%. Later, Peters & Man (1943), using the same technique with 174 cases, found a mean value of 194 mg./100 ml. with a standard deviation of 35·6 mg. Kornerup (1950), using the technique of Sperry (1936), found a mean serum total cholesterol of 218·8 ± 4·4 mg. (S.E.M.)/100 ml., S.D. 47 mg., in a series of adults.

The plasma cholesterols found in the present series of 200 normals, mean 197 ± 4·1 mg. (S.E.M.)/100 ml., S.D. 39 mg., compare favourably with these figures from the point of view of accuracy and agree also in terms of distribution, but, since plasma has been employed, tend relatively to be slightly higher than the first two sets of figures quoted above.

The evidence produced in earlier sections is of general application, and stresses the importance of the strictest control of conditions if reproducible results are to be obtained by any method of cholesterol determination employing the Liebermann-Burchard reaction.

Although plasma cholesterol levels have been estimated in the series quoted here, the author is of the opinion that until a satisfactory explanation is obtained for the differences between serum and plasma values the former should be estimated in preference to the latter, since the serum values have here been found to remain the more constant over a period of 24 hr.

SUMMARY

1. Factors influencing the colour development in the Liebermann-Burchard reaction for the determination of cholesterol have been investigated. The alteration in the densities of the absorption bands with temperature have been studied, and a simple relationship established for the conditions described.

2. A method for the estimation of serum or plasma total cholesterol suitable for routine clinical work has been evolved on the basis of the above findings, measuring the yellow component of the colour and employing a filter having a maximum transmission at 430 mμ.

3. From a series of 200 normals a mean plasma total cholesterol of 197 ± 4 mg. (S.E.M.)/100 ml., S.D. 39 mg., has been found.

The author wishes to acknowledge the valuable assistance given by Dr J. Wallace, Director of the Regional Blood Transfusion Service, in the collection of small samples of normal blood and also the encouragement and advice given by Dr J. C. Eaton and Dr I. A. Anderson at different stages of this work.

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A Micro Glass Electrode

By E. F. HARTREE
Molteno Institute, University of Cambridge

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Glass electrode assemblies as normally supplied by manufacturers require that the glass electrode, as a small bulb, together with either the standard calomel electrode or a potassium chloride-agar bridge, shall dip into the liquid under examination. Under these conditions a reduction in the volume of liquid below 5–10 ml. means that the containing vessel becomes so small that the risk of scratching the thin glass bulb becomes considerable. Rather smaller volumes can safely be used in the Morton (1930) electrode since only the glass electrode dips into the liquid; contact with the calomel electrode being through a stopcock at the base of the electrode vessel.

Most adaptations of the glass electrode to very small volumes have involved a reduction in the area of the glass membrane. Thus Païc (1938) fused a thin septum of glass at a short distance from one end of a narrow U-tube so forming a very small cup in which 0·1 ml. fluid was sufficient to cover the membrane. Chinoy (1947) made use of an eccentrically blown bulb with a depression at its uppermost point in which 0·05 ml. was placed. In both cases a bridge was used to make contact with the calomel electrode. Such electrodes naturally have a much greater electrical resistance than a fully immersed standard type of bulb, and there is again risk of damaging the membrane with the bridge when the depth of fluid is very small.

Other workers, for example Dietz (1948), have used a block of insulating material in which are two depressions connected by a small channel. The depressions are designed to fit the two electrodes so that each is surrounded by a film of liquid. The smaller the volume of liquid available for such a set up the greater are the chances of damage to the glass bulb as it is lowered into its cavity. By fitting a conventional bulb type electrode concentrically within a wider tube to allow about 1 mm. clearance Hanes (1951) could measure the pH of 1 drop of fluid which was retained in the annular space by capillarity. Such an electrode would be safe from damage as long as it remained rigidly fixed within the outer tube. Britton (1942) has reviewed various types of glass electrode.

Michaelis (1936) designed an electrode in which the glass membrane took the form of a thin-walled tube fused within a wider vertical tube. Such a design ensures complete protection of the glass membrane from mechanical damage. The centre tube held 3–5 ml. of the liquid under examination, while the annular space contained dilute hydrochloric acid into which dipped the silver-silver chloride electrode. At the lower end of the tube was fitted a 5-way stopcock to allow connexion via a potassium chloride bridge to the calomel electrode. Pickford (1937) has described a modification of Michaelis's design, for much smaller volumes (approx. 0·1 ml.). The present design is based upon that of Michaelis but is simpler than the two just mentioned and can be used with 0·3 ml. liquid. Measurements can be made very rapidly

and, if necessary, the sample can be recovered quantitatively. Furthermore, the making up of the electrode requires no great skill in glass-blowing.

Details of construction

All glass tubing was cleaned before use in sulphuric-chromic acid mixture followed by 6 N-HCl.

Since difficulty was experienced in obtaining a sound internal seal between soda glass and the inner tube of high conductivity electrode glass, short lengths of soda glass were interposed between the seals and the capillary of electrode glass.

A 2 cm. length of Corning 015 glass tubing* (8 mm. external diameter, 0·7 mm. wall) was sealed at each end to longer pieces of soda glass of the same diameter. With a glass-cutting knife a ring was scratched round each of the soda glass pieces about 8 mm. from the union with the electrode glass. Using a very narrow flame (e.g. fishtail burner) the centre of the piece of electrode glass was heated and pulled out into a thin-walled capillary about 20 cm. long and 0·5–1·0 mm. diameter. The soda glass was then cut off at the scratch marks and the ends of the tube opened out slightly (Fig. 1). If the capillary is narrower it becomes very fragile and the rate of draining would in any case be inconveniently slow.

The outer tube of the assembly was a length of soda glass about 15 mm. diameter with a curved side-arm of 6–7 mm. internal diameter. The capillary was sealed into this tube as shown in Fig. 2 and a stopcock attached to the lower end. The capillary and annular space were filled with 0·1 N-HCl and left standing 24 hr.

By measuring the resistance of the electrode membrane it is possible to reject at this stage electrodes which are obviously unsuitable, either because the membrane is too thick or because it is not continuous and the resistance therefore very low. Electrodes with resistances of 20–30 MΩ. give satisfactory results when used with a Cambridge pH meter or similar instrument. The resistance of an electrode was conveniently measured by connecting it in series with a fixed resister of 50 MΩ., a sensitive mirror galvanometer and a 2 V. battery. Connexions with the acid on each side of the membrane were made with platinum wires. If the galvanometer deflexions obtained before and after short-circuiting the electrode are d and D respectively the resistance of the electrode is $50\ (D-d)/d$ MΩ.

The side arm on the apparatus serves to hold the Ag-AgCl electrode, and for connexion of this electrode to the pH meter the co-axial cable (5·5 mm. diameter) used for television aerial leads is suitable. The outer insulation and the copper screen immediately beneath it were stripped back about 3 cm. at one end of a suitable length of cable to expose the white polythene layer. The latter was then stripped to expose about 1·5 cm. of the central copper wire to which was attached a spade terminal (or other suitable connector for the negative electrode terminal on the meter). Since the central wire is thin it is advisable to wind it back over the polythene insulation (Fig. 3*e*) and then grip it in the spade terminal. To the outer screen was soldered a piece of copper wire of a length sufficient to reach the 'earth' terminal on the meter when the spade terminal is connected.

* Other types of electrode glass would no doubt prove satisfactory.

To prevent flexing of this wire and possible breakage a short length of polythene sleeving was slipped over the wire and by gentle heating this was moulded to the polythene sleeving in the cable (Fig. 3*f*). The central wire was similarly bared at the other end of the cable (Fig. 2*c*) and soldered to a 5 cm. length of silver wire (Fig. 2*b*). When tested with a 'Megger' the resistance between the the central wire and the screen should be infinity. The silver-copper joint was covered with sealing wax and the Ag-AgCl electrode prepared by electrolysing N-HCl with the silver wire as anode. Current was passed for 3–5 min. from a 2 V. battery.

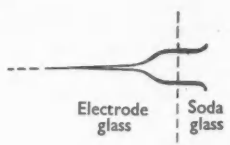

Fig. 1.

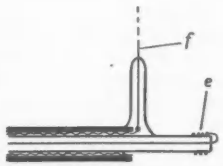

Fig. 3.

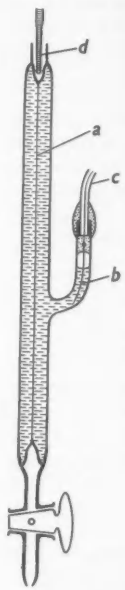

Fig. 2.

Fig. 1. Details of one end of electrode capillary.

Fig. 2. The completed glass electrode. (*a*) Capillary to hold liquid under test. (*b*) Ag-AgCl electrode dipping into 0·1 N-HCl. (*c*) Co-axial cable, the screening conductor of which is earthed. The cable is held in place with sealing wax. (*d*) Tip of KCl-agar bridge to calomel electrode.

Fig. 3. End of co-axial cable connecting with pH meter. (*e*) Central conductor wound back over polythene insulation preparatory to fixing spade terminal. (*f*) Wire connecting the screen, which surrounds the polythene insulation, to the 'earth' terminal on the pH meter.

The glass electrode was emptied, and the outer tube refilled with 0·1 N-HCl so that the acid came to within 3–4 cm. of the top of the side arm. The Ag-AgCl electrode was supported in a clamp so that it made contact with the HCl in the electrode and a test was carried out as described below. If it was satisfactory the Ag-AgCl electrode was fixed into the side arm with sealing wax so that only the silver wire was exposed beneath the seal. In a second test the 'Megger' should again read infinity.

Calomel electrode for use with the glass electrode

A convenient type of calomel electrode assembly consisted of a short test tube (10 × 2·5 cm. diameter) carrying in a two-hole rubber bung a calomel electrode (Cambridge Instrument Company) and an inverted U-tube filled with agar-KCl. The test tube contained saturated KCl plus excess of solid KCl and the end of the U-tube outside the test tube was drawn out to a capillary (2 mm. external diameter). The assembly was clamped so that the tip of its capillary was a few mm. above the upper end of the electrode capillary (Fig. 2*d*).

Testing the electrode

The leads from the calomel and Ag-AgCl electrodes were connected to a Cambridge pH meter (Cambridge Instrument Company), and the latter was adjusted after rinsing the glass electrode with standard phthalate buffer (pH 4·00) and filling with the same buffer. The pH's of a range of buffers were then determined and compared with the results obtained using the glass electrode assembly supplied by the makers of the meter. The capillary electrode was considered to be satisfactory if the results agreed within 0·02 pH units within the range 2–9. On introducing 0·3 ml. liquid at the top and closing the stopcock as soon as the liquid reached the lower end of the capillary, ample liquid remained above the capillary to ensure a junction with the agar bridge. If the tip of this bridge is < 2 mm. diameter it is impossible for it to reach and possibly to damage the narrow part of the electrode capillary.

SUMMARY

The making of a simple and robust glass electrode for pH measurements on 0·3 ml. liquid is described.

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Changes in the Protein Composition of Chick Muscle During Development

By D. S. ROBINSON
Biochemical Laboratory, University of Cambridge

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Although the earliest systematic investigations of the proteins of muscular tissue date from the work of Fürth (1895) we still know very little concerning the composition of muscles other than those of the normal adult animal. At the present time there exists a formidable array of facts and theories relating to the composition, detailed structure and functioning of adult skeletal muscle and yet, apart from a few isolated papers in the literature, little biochemical work has been carried out upon the developing tissue. Thus, the information which is available is due largely to the work of the embryologist, cytologist and histologist and is therefore mainly descriptive in character.

The changes occurring in the appearance of the muscle cell from the myoblast to the adult myofibril are reasonably well known. In the chick, with which this study is concerned, fibres can be observed in the premuscular mass of the limb buds on the 6th day of incubation which, by the 8th day, have become striated and contractile (Hunt, 1932). In the adult, the muscle fibres are composed of numerous myofibrils of diameter about 1 μ. embedded in a viscous medium, the sarcoplasm. Connective tissue fibres of collagen and elastin ramify throughout the muscle. The cell nuclei lie under the sarcolemma, having moved there during development, from a central position in the fibre.

The intracellular proteins of the adult tissue may be conveniently divided into the proteins of the sarcoplasm, such as myogen, myoalbumin, and globulin X, which together contain all the enzymes of the glycolytic cycle; and the proteins of the myofibril, such as actin and myosin. The former are extracted from minced muscle by dilute salt solutions at a neutral pH and are characterized by their distinctive physicochemical properties. The myofibrillar protein, actomyosin, is extracted only by strong salt solutions and precipitates on dilution. If extraction is prolonged at an alkaline pH then the actin content of the extract rises and precipitation on dilution yields an actin-rich actomyosin complex.

The protein tropomyosin (Bailey, 1948), while also probably located within the myofibril, can only be extracted by special methods.

Remaining after strong salt extraction is a residue which is partly soluble in dilute acid. Bate Smith (1937) thought that the acid-soluble part consisted largely of denatured myosin and globulin X and belonged to the intracellular fraction, while the acid-insoluble residue consisted of collagen, elastin and reticulin which together formed the extracellular fraction.

Such studies on the solubilities and distribution of the muscle proteins of the adult have led to the development of schemes for their fractionation such as those used by Weber & Meyer (1933) and by Bate Smith (1934, 1935, 1937). In this paper a similar scheme has been applied to the developing tissue and the quantitative relations between certain groups of muscle proteins have been determined. Such a survey seems an essential preliminary to any more detailed work on the biochemistry of muscle development. Subsequent papers describe the nucleoprotein and adenosinetriphosphatase distribution in the developing tissue (Robinson, 1952*a*, *b*).

EXPERIMENTAL

The solubility characteristics of the proteins of adult striated muscle

Bate Smith (1934, 1935) has demonstrated that the extraction of minced adult rabbit muscle with strong salt solutions at neutral pH brings into solution the major part of the myofibrillar and sarcoplasmic proteins, though the discrepancy between the amount extracted by this procedure and that using 0·1 N-HCl may amount to 15% of the total intracellular protein. It is probable that some of the actin is not extracted by neutral salts under Bate Smith's conditions, but is soluble in 0·1 N-HCl.

The myofibrillar proteins are precipitated from such a salt extract at pH 7 by dilution to an ionic strength (I) of 0·05; globulin X and the other sarcoplasmic components remain in solution. According to Bate Smith, however, the completeness of the precipitation depends upon the nature of the ions present. He was able to estimate the amounts remaining in solution under different conditions and found, for example, that by 20-fold dilution of a 7% (w/v) LiCl extract only 88% of the myofibrillar proteins were precipitated. Similarly, it was found by the author that dilution of an actomyosin preparation in 1·25 M-KCl-phosphate buffer to an ionic strength of 0·1 did not bring about complete precipitation of the protein. Dilution to an ionic strength of 0·05 would probably have further reduced the amount remaining in solution, but the volume of liquid to be handled would have become too large for the routine fractionations which were contemplated.

Work in a related field (Cohn *et al.* 1946) on the fractionation of plasma proteins, using ethanol at low temperatures as the precipitating agent, has indicated that fibrinogen, a protein similar in its solubility to actomyosin, is precipitated from plasma at pH 7 by an ethanol concentration of 7% (v/v) at −3°. It seemed possible that a similar system could be used for the precipitation of actomyosin. Experiments carried out on a pure preparation of rabbit actomyosin in strong KCl-phosphate, showed that, on dilution to an ionic strength of 0·12 and addition of 70% (v/v) ethanol from a fine capillary to the stirred preparation at 0°, all the protein was precipitated at an ethanol concentration of 7% (v/v). Using artificial mixtures of an actomyosin preparation and a sarcoplasmic extract of adult rabbit muscle, actomyosin could be quantitatively recovered under the same conditions with an error of ±5%. With a sarcoplasmic extract in dilute KCl-phosphate, addition of ethanol to 20% (v/v) gave no precipitation of any protein component.

The effect of different salt solutions upon the extraction of muscle proteins

Bate Smith (1934) concluded from a survey of various salts that 7% (w/v) LiCl solution at pH 7 extracted the largest percentage of the intracellular proteins from minced muscle. A similar study was carried out using the newer extraction techniques described in detail below, but at pH 8·5 rather than at pH 7 to facilitate the solution of the protein, actin. The results of three such experiments are given in Table 1.

Table 1. *The effect of different salts upon the extraction of the muscle proteins*

Extractant	Protein N extracted (g./100 g. fresh muscle)
0·5 M-KCl in 0·066 M-phosphate buffer pH 8·5	2·07
1·25 M-KCl in 0·066 M-phosphate buffer pH 8·5	2·38
7% LiCl in 0·05-M-borate buffer pH 8·5	2·45

The efficiency of the 1·25 M-KCl-phosphate solution at pH 8·5 seems to be very little below that of the 7% (w/v) LiCl-borate solution and because of the rapid destruction of double refraction of flow of myosin which occurs in the presence of the latter, according to Edsall & Mehl (1940), the strong KCl-phosphate solution has been preferred in this study.

The residue remaining after extraction with a strong KCl-*phosphate solution*

This fraction contains approximately 20–25% of the total protein of adult rabbit muscle. When extracted with 0·1 N-NaOH much more protein material goes into solution leaving a residue forming about 5% of the total protein. This is believed to consist only of collagen and elastin, since it has been shown (Lowry, Gilligan & Katersky, 1941; Bowes & Kenten, 1948) that while other tissue proteins are soluble these two are insoluble in 0·1 N-NaOH solution.

The state of division of the tissue

Many workers have stressed the importance of the state of dispersion of the tissue in the extraction of the intracellular proteins (Bate Smith, 1937; Guba & Straub, 1943; Dubuisson, 1950). Experiments were made with a Latapie mincer followed by a glass homogenizer, a Waring Blendor, a treatment which involved grinding with sand after mincing with

scissors, and with a stainless-steel homogenizer, described by Marsh & Snow (1951). The last was found to be the most satisfactory for adult muscle, and in less than a minute reduced it to disintegrated and broken myofibrils.

There appears to be no denaturation of the sarcoplasmic proteins in the homogenizer when running at top speed in the presence of either the strong KCl-phosphate solution or the dilute KCl-phosphate solution. The small amount which occurs when actomyosin solutions are similarly treated at 0° does not amount to more than 4% of the total protein.

Fractionation procedures

The studies already described suggest certain factors which should be considered in any proposed fractionation. It seems clear that the state of division of the tissue, the pretreatment of the muscle, the time and conditions of the extraction (pH, temperature, etc.) and the nature of the salts used are important in obtaining a quantitative recovery of any particular fraction.

overnight in 0·1 N-NaOH at 0°. The extract was discarded and the residue dissolved in concentrated H_2SO_4 and samples taken for protein N determination. The scheme is summarized in Fig. 1.

Protein N was estimated by the micro-Kjeldahl method (Chibnall, Rees & Williams, 1943), but 15 ml. lipped Pyrex test tubes were used instead of the customary 50 ml. digestion flasks. Thus the precipitation of the protein, the washing of the precipitate and the digestion with H_2SO_4 could be carried out in the same vessel. All micro-Kjeldahl estimations were made in triplicate: at the earliest developmental stages where the amount of protein N/sample was less than 9·2 mg. a Markham apparatus was used instead of the usual distillation system and the subsequent titration carried out with a 1 ml. horizontal burette.

Extraction with strong salt solution. Weighed samples of fresh muscle were treated as above, except that a stronger salt solution of composition 1·25 M-KCl, 0·066 M-K_2HPO_4 pH 8·5, $I = 1·45$ was used. This will be called the strong KCl-phosphate solution. The combined salt extracts were

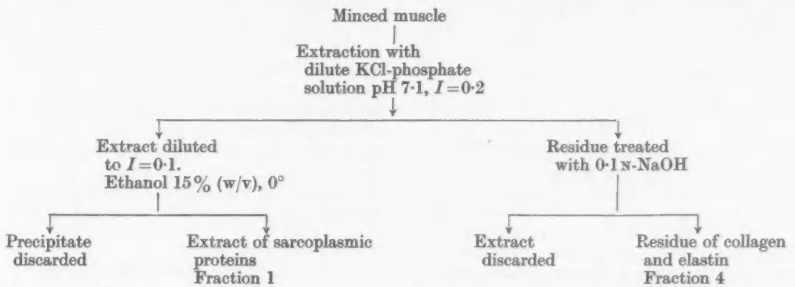
Minced muscle
Extraction with dilute KCl-phosphate solution pH 7·1, I = 0·2
Extract diluted to I = 0·1. Ethanol 15% (w/v), 0°
Residue treated with 0·1 N-NaOH
Precipitate discarded
Extract of sarcoplasmic proteins Fraction 1
Extract discarded
Residue of collagen and elastin Fraction 4

Fig. 1. Scheme showing the fractionation achieved with dilute KCl-phosphate and 0·1 N-NaOH solutions.

The procedures we have used are based on extraction with three different solutions which the preliminary studies suggest will yield four distinct fractions of muscle proteins. The details are given below.

Extraction with a dilute salt solution. Weighed samples of fresh muscle (from 1 to 3·5 g.) were finely minced with scissors in the cooled extractant solution. This had the composition, 0·1 M-KCl, 0·066 M-NaH_2PO_4/K_2HPO_4 pH 7·1, $I = 0·2$, and will be referred to as the dilute KCl-phosphate solution. The whole was transferred to the homogenizer which was never more than half full of solution (capacity 50 ml.) and was surrounded by an ice bath. A drop of 'capryl alcohol' was added to reduce frothing. After homogenization for 5 min. at top speed, the homogenizer, designed to fit into a 50 ml. centrifuge cup, was centrifuged and the supernatant solution poured off. Second and third extractions for 10 min. at half speed were carried out in a similar fashion. The three extracts were combined and samples diluted at pH 7 with 30% ethanol (v/v) to $I = 0·1$ at an ethanol concentration of 15% (v/v). The ethanol was added from a fine capillary to the stirred extract at 0°. The small precipitate of actomyosin which formed was removed on the centrifuge and the protein N content of the supernatant solution determined.

The residue remaining after extraction with dilute KCl-phosphate was extracted twice with 0·1 N-NaOH for periods of 10 min. in the homogenizer at half speed and then stood then diluted with 0·066 M-NaH_2PO_4 and water to a final KCl concentration of 0·5 M and an ionic strength of 0·58 at pH 7·1. Samples of this extract were further diluted in the Pyrex tubes with 19% ethanol (v/v) to an ionic strength of 0·12 and a final ethanol concentration of 15% (v/v). The temperature was kept at 0° throughout the addition. The precipitated protein was centrifuged and washed twice with 5% trichloroacetic acid (w/v) before determination of the protein N content.

The residue after extraction with the strong KCl-phosphate solution was treated with 0·1 N-NaOH as previously described, and both the NaOH extract and residue retained for nitrogen determination. The scheme is summarized in Fig. 2.

In addition, it was usual, as a check upon the analyses of the fractions, to determine the total protein N directly. Weighed samples of muscle were minced with 10% trichloroacetic acid (w/v) and, after centrifuging and washing, the residue was dissolved in concentrated H_2SO_4 before samples were taken for final digestion.

A summary of the fractions estimated. The protein fractions determined are, therefore:

Fraction 1. Soluble in dilute KCl-phosphate at pH 7·1 and not precipitated on dilution to an ionic strength of 0·1 at an ethanol concentration of 15% (v/v) at 0° (Fig. 4*A*).

Fraction 2. Soluble in strong KCl-phosphate at pH 8·5, but precipitated on dilution to an ionic strength of 0·12 and

a final ethanol concentration of 15% (v/v) at pH 7·1 at 0° (Fig. 4*B*).

Fraction 3. Insoluble in strong KCl-phosphate at pH 8·5, but soluble in 0·1 N-NaOH (Fig. 4*C*).

Fraction 4. The residue after extraction with 0·1 N-NaOH (Fig. 5*A*).

Fraction 5. Total protein N (Fig. 5*B*).

In the later stages of development after hatching, when all the muscle was not required, samples as representative as possible were taken. Analyses carried out on three such samples, taken from the breast muscle of a single month-old chick, agreed to within 5%. Consequently, the larger variations when muscle was taken from different birds of the same weight must be attributed to general environmental

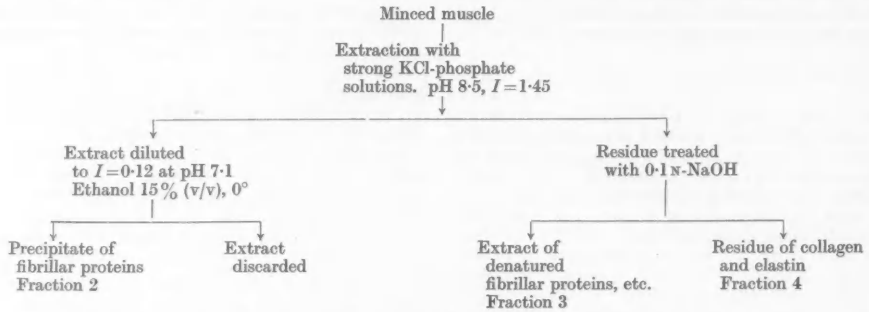
Minced muscle
Extraction with strong KCl-phosphate solutions. pH 8·5, I = 1·45
Extract diluted to I = 0·12 at pH 7·1 Ethanol 15% (v/v), 0°
Residue treated with 0·1 N-NaOH
Precipitate of fibrillar proteins Fraction 2
Extract discarded
Extract of denatured fibrillar proteins, etc. Fraction 3
Residue of collagen and elastin Fraction 4

Fig. 2. Scheme showing the fractionation achieved with strong KCl-phosphate and 0·1 N-NaOH solutions.

Materials

Incubation. The eggs, incubated in a standard incubator or in a hot room at 37°, were from a single strain of White Leghorns. The description of an embryo's stage of development in terms of the length of time of incubation is far from satisfactory, and the weight of the embryo or chicken has been used as a better, and easily available, measure of the development state. The relation between age and weight of the chick embryo is shown in Fig. 3 (data from Romanoff, Smith & Sullivan, 1938).

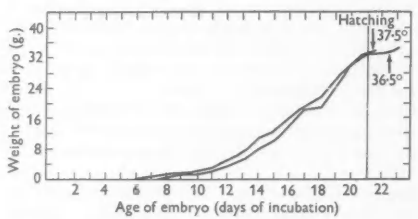

Fig. 3. The relation between age and weight of the chick embryo at incubation temperatures of 37·5 and 36·5° (average values for 700 White Leghorn embryos, Romanoff *et al.* 1938).

Dissection techniques. The analyses covered a range from the chick embryo of 13 days' incubation, through the hatching period around 21 days' incubation, to the adult bird. A routine method of dissection was devised and adhered to throughout. The egg was broken and the embryo removed, dried lightly on filter paper, weighed rapidly and immediately dissected. The muscle from each breast was removed as completely as possible and transferred to the appropriate extracting solution contained in a weighed vessel at 0°.

factors and a comparatively large number of analyses had to be carried out, therefore, on the older birds. In the earlier stages where at least 1 g. of muscle was required for a complete fractionation and, consequently, several embryos had to be harvested, the variations were less. The facilities and help given by Dr D. B. Cater are gratefully acknowledged.

RESULTS

In Figs. 4*A–C* and 5*A, B* the protein nitrogen content of each fraction has been expressed as g./100 g. fresh weight of muscle. In Fig. 6 the individual curves are shown together to facilitate comparison (the individual determinations are not given). In Fig. 7 the protein nitrogen of the different fractions is expressed as a percentage of the total protein nitrogen. The breaks which occur in all the curves at hatching are most probably due to changes in the water content of the tissue and have not been studied in detail.

Fraction 1 consists of sarcoplasmic proteins, including many of the enzymes of the glycolytic cycle, and the particulate components of the cell cytoplasm. As a percentage of the fresh weight it steadily increases during growth though it forms a rapidly decreasing proportion of the total protein throughout the embryonic period. It reaches a steady value about a fortnight after hatching.

Fraction 2 consists largely of the proteins of the muscle fibril. However, the deoxyribonucleoproteins of the cell nuclei will form a considerable proportion of the whole in the embryonic stages since, like the myofibrillar proteins, they are extracted by strong salt solutions and precipitated on dilution. Their contribution is assessed in a subsequent paper

(Robinson, 1952*a*). The fraction appears to increase throughout the period over which the analyses have been carried out, both as a percentage of the fresh weight and of the total protein, though the rate of increase declines about a fortnight after hatching.

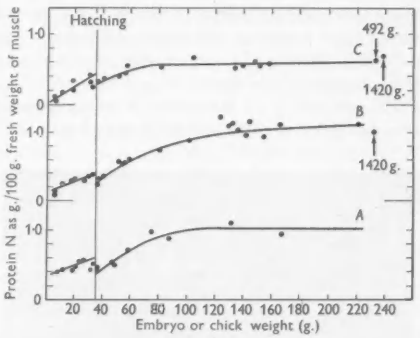

Fig. 4. Changes in the protein nitrogen content of certain muscle fractions during embryonic development; expressed as g./100 g. fresh weight of muscle. *A*, protein nitrogen soluble in dilute KCl-phosphate at pH 7 and not precipitated on dilution to an ionic strength of 0·1 at an ethanol concentration of 15% (v/v) (Fraction 1). *B*, protein nitrogen soluble in strong KCl-phosphate at pH 8·5, but precipitated on dilution to an ionic strength of 0·12 and a final ethanol concentration of 15% (v/v) at pH 7 (Fraction 2). *C*, protein nitrogen insoluble in strong KCl-phosphate at pH 8·5, but soluble in 0·1 N-NaOH (Fraction 3).

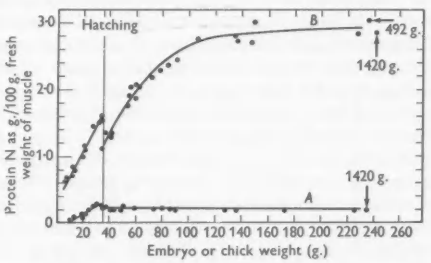

Fig. 5. Changes in the protein nitrogen content of certain muscle fractions during embryonic development; expressed as g./100 g. fresh weight of muscle. *A*, protein nitrogen insoluble in 0·1 N-NaOH (Fraction 4). *B*, total protein nitrogen (Fraction 5).

Fraction 3 is complex and almost certainly contains unextracted actomyosin, either in the native or denatured form. In addition, however, the ill-defined 'ground substance' is probably a component in the embryonic stages where it forms, together with the extracellular proteins, a structural basis upon which the cell architecture is built. Certainly its importance in growing and differentiating tissues should not be minimized and it is interesting to note the large proportion of the total protein of the muscle which the fraction forms during the earlier periods of development. At hatching it represents as much as 25% of the total protein and, therefore, at this stage the residual proteins after extraction with strong salt (Fractions 3 and 4) form almost 50% of the total protein of a muscle which is apparently fully functional. As a percentage of the fresh weight, the fraction appears to increase more

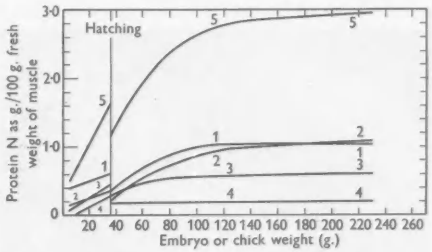

Fig. 6. The protein nitrogen of Fractions 1–5, expressed as g./100 g. fresh weight of muscle at different developmental stages.

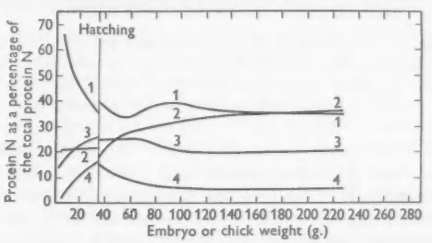

Fig. 7. The protein nitrogen of Fractions 1–4, expressed as percentage of the total protein nitrogen (Fraction 5) at different developmental stages.

rapidly than the other fractions during the embryonic stages; after hatching its increase is less marked, and as a percentage of the total protein it decreases to a constant, yet still high, level about a fortnight after hatching. Here it probably gives a better measure of the unextracted actomyosin of the muscle.

The mucopolysaccharides extracted from muscle by Dische & Osnos (1950) would probably be located in this fraction, which from a brief histochemical study does appear to contain such material. Part of the fraction is soluble in urea and its solutions in 0·1 N-sodium hydroxide have a high viscosity which, however, falls on standing, possibly due to depolymerization. The fraction will shrink in the presence of adenosinetriphosphate suggesting that some actomyosin is present, although strong salt

solutions will not dissolve the precipitate either before or after syneresis.

Fraction 4 increases as a percentage of the fresh weight until hatching and then forms a relatively constant percentage throughout post-embryonic growth. It contributes 15% of the total protein nitrogen of the muscle at hatching and appears to be the first component to be fully differentiated. As development proceeds it forms less and less of the total protein, falling to a constant level, about 5% of the whole, a month after hatching.

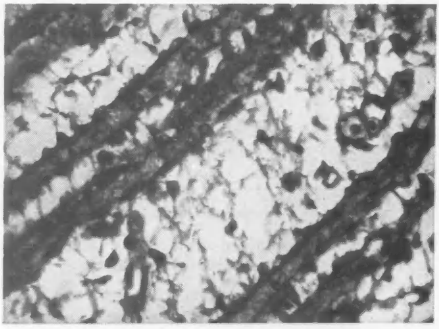

Fig. 8. A section of 13-days'-incubated embryonic chick muscle stained with haematoxylin and eosin. Magnification ×660. Note the developing muscle fibrils with centrally placed nuclei and the 'reticulin' network.

The fraction is made up of the extracellular proteins, collagen, elastin and reticulin. Histologically, the young embryonic muscle shows a vast interlacing network of fibrils which from their staining properties appear to consist largely of reticulin. They stand out very clearly in sections (Fig. 8) and may well form a structural framework for myofibril development, thus accounting for the relatively early differentiation of the fraction. In the adult the histologists suppose it probable that reticulin becomes converted to collagen, and certainly a stained section of the adult muscle shows no such obvious ramifying network as exists in the embryo (Fig. 9). The author is indebted to Mr D. Canwell of the Physiology Department, Cambridge, for these preparations.

In Fraction 5 the total protein nitrogen as a percentage of the fresh weight increases steadily throughout development, reaching its maximum value when the chick is about a month old. It is merely the resultant curve of the changes observed in the other fractions analysed and cannot yield very much information concerning the structural differentiation of the tissue.

It is interesting to contrast the analyses which have been presented with those of Bate Smith (1937)

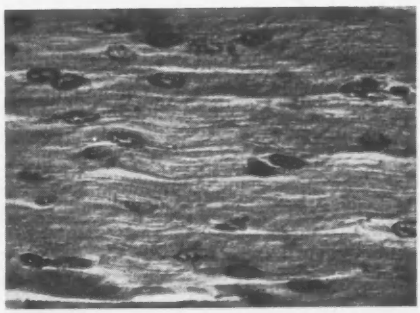

Fig. 9. A section of adult fowl muscle, stained with haematoxylin and eosin. Magnification ×880. Note the striated myofibrils and elongated nuclei. The 'reticulin' network has disappeared.

and Hasselbach & Schneider (1951) on adult rabbit muscle. In Table 2, protein analyses of the muscles of month-old and newly hatched chicks are presented together with those of adult rabbit muscle. In the month-old chick the muscle has acquired its adult form and the partition of nitrogen between the fractions has reached a steady level.

The true contribution of the extracellular proteins appears to be less than Bate Smith (1937) and Hasselbach & Schneider (1951) found in the rabbit. However, it must be emphasized that the extracellular fractions are not directly comparable in these analyses. Thus, in the analyses of Bate Smith they have been calculated from extractions with

Table 2. *Analyses of chicken breast muscle and adult rabbit striated muscle expressed as percentages of the total protein*

Description of fraction	Chicken breast muscle (immediately after hatching)	Chicken breast muscle (1 month after hatching)	Adult rabbit striated muscle	
			Bate Smith	Hasselbach & Schneider
Intracellular:				
Myofibrillar protein	17·5 } 42·5	40·0 } 62	53·6 } 68·6	53–55
Residual protein	25·0 }	22·0 }	15·0 }	
Sarcoplasmic protein	40·0	33·0	16·4	28
Extracellular:				
Collagen, elastin, reticulin	17·5	5·0	15·0	15–17

dilute acid and in those of Hasselbach & Schneider (1951) from extractions with urea, whereas in our fractionation dilute sodium hydroxide has been used. Certainly neither 0·1N-hydrochloric acid nor urea solutions extract from chick muscle as large a percentage of the total protein as 0·1N-sodium hydroxide and it seems that this last leaves a residue which consists solely of collagen, elastin and reticulin. These factors as well as species differences may be, in part, responsible for the differences in the analyses.

The high extracellular fraction in the young chick has already been commented upon. Except for this extracellular fraction the analytical values during development appear to be tending towards those of the adult rabbit muscle.

DISCUSSION

The results have shown marked differences in the rates of increase of the protein fractions, and, moreover, that the rates vary in themselves during development. From the form of the curves presented it is difficult to decide whether there is any interchange between the fractions. For example, expressed as a percentage of the total protein, the sarcoplasmic proteins (Fraction 1) decrease during the embryonic stages while the myofibrillar fraction (Fraction 2) is increasing. It may be that protein is being laid down in the developing fibril, having been preformed in the sarcoplasm. This concept will receive added point in a subsequent paper when the adenosinetriphosphatase activities of these two fractions are considered (Robinson, 1952*b*).

Similar work to that described has been carried out by Hermann & Nicholas (1948) on the muscle proteins of growing rats. Their analyses are similar to those given here, although they did not attempt to estimate the true collagen, elastin and reticulin content of the residue after strong salt extraction, though they appreciated its probable complexity. The time relations of their curves differ from ours somewhat, but that is understandable since the rat at birth is at an earlier stage of general development than is the newly hatched chick. In the rat the residue left after extraction with strong salt solutions, when expressed as a percentage of the fresh weight, reaches a high value about 15 days after birth and then falls to a lower value in the adult. This has no counterpart in the chick where the comparable fraction (Fractions 3+4) rises to a high value at an earlier stage about 10 days after hatching, and thereafter forms a constant percentage of the fresh weight of the muscle. During the development of the rat the sarcoplasmic fraction (Fraction 1) forms a lower percentage of the total protein than it does in the chick, while the rate of increase of the myofibrillar fraction (Fraction 2) after birth is much greater than it is after hatching.

Throughout this work one of the primary aims has been to obtain a quantitative recovery of each fraction and no attempt has been made to isolate any protein component of the muscle in the pure state. A single estimation of the tropomyosin content of embryonic chick muscle has, however, been made in view of Bailey's (1948) suggestion that tropomyosin might function as a prototype of the myosin molecule. If this were so it might occur in large amounts in embryonic tissues where the elaboration of the fibril is taking place. However, in embryos incubated for 16 days, it formed only about 1% of the total protein of the muscle, an amount comparable to that found in the adult.

So far as any individual protein is concerned, analyses of the type reported can throw little light on its behaviour. All the fractions which have been estimated are complex and doubtless contain several distinct proteins. However, the fractionation can form the basis for more detailed work on the interchange between the protein components. In addition, it is an essential to any further work on such individual components and it is perhaps in that direction that it will be of most value. In particular those proteins which may be characterized easily by their physicochemical properties could be studied in relation to the fractionation scheme as a whole. One such example, tropomyosin, has already been mentioned.

A further examination of the residue remaining after strong salt extraction would be extremely interesting. Study of the poorly defined 'ground substance' is urgently needed since a detailed knowledge of its nature, both as a matrix in which particular cell structures are elaborated, and possibly as a source for their elaboration, would be extremely valuable at the embryonic level where differentiation is proceeding.

SUMMARY

1. A study has been made of the quantitative changes occurring in the protein composition of the chick breast muscle as the tissue develops from the embryonic to the adult state.

2. A standard method for the extraction and estimation of several protein fractions has been developed. The sarcoplasmic proteins extracted from the homogenized muscle tissue by dilute salt solutions, and the fibrillar proteins extracted in strong salt solutions but precipitated on dilution, form two such fractions. That protein fraction insoluble in strong salt solutions but soluble in dilute alkali forms a third complex fraction and the final extracellular residue, a fourth.

3. The changes found in the protein nitrogen content of each fraction during the development of

the chick have been presented with reference both to the fresh weight of the tissue and the total protein. Protein analyses of the muscles of month-old and newly hatched chicks are contrasted with those of adult muscle from the rabbit.

The author is indebted to the Medical Research Council for a Studentship held during the course of this work. He would also particularly like to thank Dr K. Bailey for his guidance throughout and Dr D. B. Cater, F.R.C.S., for his interest and help.

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Changes in the Nucleoprotein Content of Chick Muscle during Development

By D. S. ROBINSON
Biochemical Laboratory, University of Cambridge

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Whilst adult striated muscle is particularly well adapted to perform its specific function in the body, it nevertheless possesses certain elements, such as the nuclei and the particulate components of the cytoplasm, which are common to all cells. The quantitative contribution of these latter to the whole varies during development; it is large in the earlier embryonic stages, but falls, as the myofibrils differentiate, to a lower level in the adult muscle (Graff & Barth, 1938; Caspersson, 1941, 1947, 1950; Brachet, 1945, 1947).

In a previous paper a protein fractionation procedure has been described and applied to chick muscle at various developmental stages (Robinson, 1952). Since the nucleoproteins of the cell nucleus have solubility properties similar to those of the myofibrillar proteins (Brachet & Jeener, 1947; Mirsky & Pollister, 1942, 1943), they have been estimated with them in this scheme. Thus, whilst none of the deoxyribonucleic acid of the cell nucleus was found in dilute KCl-phosphate extracts of muscle (Fraction 1) over 80% was extracted in strong KCl-phosphate solutions and was precipitated, with the myofibrillar proteins, by 15% (v/v) ethanol on dilution to an ionic strength of 0·12 at 0° (Fraction 2). The remaining 20% of the deoxyribonucleic acid was not extracted and was estimated in the residual fractions (Fractions 3 and 4). The numerical designations of the fractions refer throughout this paper to Robinson (1952).

In this study the contribution made by deoxyribonucleoproteins of the cell nucleus has been assessed as follows. Nuclei have been isolated from chick muscle at different developmental stages and the deoxyribonucleic acid/protein ratio has been determined. Then, knowing the deoxyribonucleic acid content of the muscle residue after extraction with dilute KCl-phosphate solution (Fraction 2+3+4), the amount of deoxyribonucleoprotein in this fraction has been calculated. Furthermore, a better assessment of the myofibrillar protein content of muscle has been made by correcting the previous analyses for the newly determined nuclear protein contribution.

EXPERIMENTAL

The sources of muscle material and details of the dissection and extraction techniques have been given previously (Robinson, 1952). The nucleic acid contents of the following fractions have been determined: Fraction 1, the extract of muscle in 0·1 M-KCl, 0·066 M-NaH_2PO_4/K_2HPO_4, $I = 0{\cdot}2$, pH 7·1, and Fraction 2 + 3 + 4, the residue from the extraction of muscle with the above solution.

The terms deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) are used as convenient notations without necessarily assuming that the nucleic acid material from the muscle is identical with that from other organs.

Extraction and estimation of nucleic acids. Schneider's (1945) procedure for the extraction of nucleic acids was used without serious modification. Total nucleic acids were estimated from their purine and pyrimidine absorption in the ultraviolet, measured in the Beckman spectrophotometer (model DU), and from their organic phosphorus content determined by Allen's (1940) method. DNA was determined by a modification of the Dische (1930) method described by Davidson & Waymouth (1944), and RNA was estimated by a modification of the orcinol method of Mejbaum (1939) due to Militzer (1946), but using the period of heating recommended by Albaum & Umbreit (1947). The orcinol estimations were always corrected for the effect of any DNA which was present.

The standard nucleic acids used were kindly provided by Dr K. Bailey. The RNA was a Boehringer product from yeast and the DNA a pure sample from thymus gland. Both preparations were free from inorganic phosphorus and contained 9·60 and 9·63% organic phosphorus respectively. After treatment with hot trichloroacetic acid, as in the Schneider (1945) procedure, the ultraviolet absorption maximum of the DNA standard solution, measured against a blank solution of trichloroacetic acid, is displaced from 2600 to 2680 A. Using the notation suggested by Chargaff & Zamenhoff (1948) the atomic extinction coefficients (ϵP) for our acid-treated samples were 10 050 for RNA and 8450 for the DNA sample. The DNA value is considerably higher than that given by Vischer & Chargaff (1948) (9800 and 6056 respectively), but both are in good agreement with values quoted by Ogur & Rosen (1950) (10 800 and 8780 respectively) for samples after treatment with perchloric acid. In addition to causing the shift in maximal absorption it appears that breakdown of DNA to nucleotide units by acid treatment increases the degree of absorption in the ultraviolet.

The nucleic acid extracts were diluted to contain 10–20 μg. nucleic acid/ml. for measurements of ultraviolet absorption; 10–60 μg. nucleic acid/ml. for measurements of phosphorus content; 50–200 μg. nucleic acid/ml. for estimations by the orcinol method for RNA; 500–1500 μg. nucleic acid/ml. for estimations by the diphenylamine method for DNA.

The sum of the separate analyses for DNA and RNA in each fraction should agree with the total nucleic acid values derived from the ultraviolet absorption and the phosphorus determination; although there was reasonable agreement for the extract of muscle in dilute KCl-phosphate (Table 1), the sum was in general slightly higher in the analyses of the residue (Table 2), due possibly to the presence of mucopolysaccharide material in this fraction.

Since the only nucleic acid present in the dilute KCl-phosphate extract is RNA the ultraviolet extinction coefficient of the RNA standard was used to calculate the nucleic acid content of this fraction. In the residue, in view of the preponderance of DNA, the corresponding coefficient of the DNA standard was used.

Isolation of muscle nuclei. Nuclei were isolated at pH 4 using a citric acid method similar to that described by Dounce (1943). Differential centrifugation of the homogenized muscle (4–10 g.) yielded samples of nuclei free from sarcoplasmic and fibrillar material when examined under the microscope (Fig. 1). The nuclei seem to have retained their

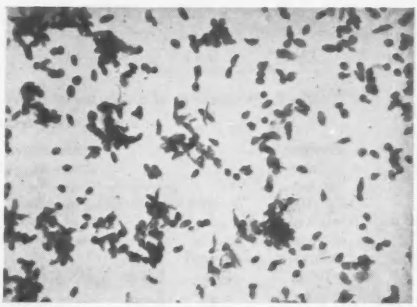

Fig. 1. Preparation of cell nuclei from muscle of embryos incubated for 16 days. Stained with haematoxylin and eosin. Magnification, ×370.

shape, appear unbroken, and in certain cases nucleoli are visible. In the adult animal, where the nuclei form a very small part of the whole muscle, greater difficulty was encountered in obtaining a preparation free from fibrillar fragments. Nevertheless, this could be achieved by using larger quantities of starting material and by sacrificing a greater proportion of the contaminated nuclei.

The amount of citric acid required to maintain the pH around 4 varied according to the state of development of the tissue and had to be determined previously by titration. Small quantities were added at intervals throughout the preparation to maintain the pH, which otherwise tended to rise and cause agglutination.

It is probable that nuclei isolated using such a method will have lost a considerable amount of protein during their preparation. Although neither nucleic acid nor the basic histone protein is likely to be soluble at pH 4, any acidic proteins of the nucleus may be (Behrens, 1932; Dounce, 1950). However, the analyses are to be referred to the residue from the extraction of the muscle with dilute KCl-phosphate and this fraction will contain nuclei subjected similarly to aqueous media. It has been assumed that the loss of water-soluble proteins is approximately equivalent in each case, although the conditions do differ particularly with respect to pH.

Nucleic acids were extracted and estimated as before. Protein nitrogen was estimated in the residue by the micro-Kjeldahl method after the nucleic acid had been extracted. No phosphorus estimations were made.

Table 1. *Nucleic acid analyses of dilute* KCl-*phosphate extracts of chick muscle*

Av. wt. of chick (g.)	Nucleic acid + protein as g./100 g. fresh weight of muscle (Robinson, 1952)	Weight of muscle analysed (g.)	Nucleic acid as g./100 g. fresh weight of muscle: Colorimetric RNA	Colorimetric DNA	Phosphorus Total nucleic acid	Ultra-violet Total nucleic acid	Nucleic acid as % of the total protein + nucleic acid of muscle: Colorimetric RNA	Colorimetric DNA	Phosphorus Total nucleic acid	Ultra-violet Total nucleic acid
3·5	2·75	1·42	0·29	—	—	0·30	11·30	—	—	11·50
7·8	3·75	3·99	0·23	—	0·27	0·22	6·18	—	7·25	5·80
8·9	3·95	5·69	0·23	—	—	0·22	5·75	—	—	5·66
13·7	5·10	6·66	0·28	—	—	0·24	5·45	—	—	4·65
16·7	5·63	8·06	0·21	—	—	0·22	3·78	—	—	3·85
23·8	7·50	4·42	0·23	—	0·24	0·22	3·02	—	3·18	2·99
Hatching 142	18·0	7·14	0·06	—	—	0·08	0·32	—	—	0·45

Table 2. *Nucleic acid analyses of the residue remaining after extraction of chick muscle with dilute* KCl-*phosphate*

Av. wt. of chick (g.)	Nucleic acid + protein as g./100 g. fresh weight of muscle (Robinson, 1952)	Weight of muscle analysed (g.)	Nucleic acid as g./100 g. fresh weight of muscle: Colorimetric RNA	Colorimetric DNA	Colorimetric RNA + DNA	Phosphorus Total nucleic acid	Ultra-violet Total nucleic acid	Nucleic acid as % of the total protein + nucleic acid of muscle: Colorimetric RNA	Colorimetric DNA	Colorimetric RNA + DNA	Phosphorus Total nucleic acid	Ultra-violet Total nucleic acid
3·5	2·60	1·42	0·040	0·21	0·250	—	0·23	1·40	8·1	9·5	—	8·80
7·8	3·75	3·99	0·050	0·19	0·240	0·23	0·21	1·32	5·1	6·42	6·25	5·55
8·9	3·95	5·69	0·025	0·20	0·225	0·18	0·22	0·63	5·1	5·73	4·62	5·52
13·7	5·10	6·66	0·023	0·20	0·223	—	0·22	0·45	4·0	4·45	—	4·27
16·7	5·63	8·06	0·033	0·14	0·173	0·17	0·19	0·59	2·5	3·09	3·04	3·41
23·8	7·50	4·42	0·046	0·22	0·266	0·23	0·21	0·61	3·0	3·61	3·10	2·85
Hatching 142	18·0	7·14	0·039	0·033	0·072	—	0·06	0·22	0·18	0·40	—	0·34

RESULTS

Nucleic acid content of extracts and residues

The nucleic acid analyses of the dilute potassium chloride-phosphate extract are given in Table 1, and the analyses of the residue after such extraction, in Table 2.

Expressed as g./100 g. fresh weight of muscle the amounts of nucleic acid remain at a fairly constant level both in the sarcoplasmic extract and in the residue throughout embryonic development. The relative constancy of the nucleic acid content for such a long period is rather surprising when it is considered in relation to the changing composition of embryonic muscle as a whole. Comparison with the analysis of muscle from a 3-week-old chick weighing 142 g. shows that a large fall in the amount of nucleic acid/g. of muscle occurs after hatching.

When the results are expressed as percentages of the total protein of the muscle (actually protein plus nucleic acid) on the basis of the analyses previously reported (Robinson, 1952), the nucleic acids in each fraction form a steadily decreasing percentage throughout embryonic development. Novikoff & Potter (1948), in a study of the nucleic acid content of the whole chick embryo during development, find a peak in the RNA and DNA content after 15 days incubation (12 g. embryos) expressed as mg./100 g. of dry tissue. The present results show that this cannot be due to changes in the nucleic acid content of the muscle tissue.

In the embryonic muscle the RNA/DNA ratio lies between 1·6 and 1·25, while in the 142 g. chick (3 weeks old) it is approximately 3·0. Davidson & Waymouth (1944), in a study of sheep muscle, find a RNA/DNA ratio equal to 1·4 in the embryo, rising to 3·6 in the adult.

Analyses of muscle nuclei

The amount of nucleic acid, expressed as a percentage of the nucleic acid plus protein, varied between 25 and 44% at different embryonic stages for seven samples of isolated nuclei. The variations may be the result of contamination or of the extraction of varying amounts of nucleoprotein during the isolation. Analyses in the literature for the isolated nuclei of other tissues vary widely between

20 and 60% (Mirsky & Pollister, 1942; Dounce, 1943; Mirsky & Ris, 1949). The RNA/DNA ratio varied between 0·1 and 0·2, in good agreement with values in the literature (Caspersson, 1941, 1947, 1950; Davidson & Waymouth, 1944; Vendrely & Vendrely, 1948).

The nucleoprotein and myofibrillar protein content of the residue after extraction with dilute potassium chloride-phosphate

Nucleoprotein content. Using the ultraviolet absorption data of Table 2, and taking an average value of 0·33 for the ratio nucleic acid/nucleic acid plus protein in the embryonic nucleus, the changes in the nucleoprotein content of the residue after extraction with dilute KCl-phosphate solutions can be represented in relation to the fresh weight and total protein plus nucleic acid content of the muscle (latter data derived from values in Robinson, 1952). This has been done in the curves *B* of Figs. 2 and 3.

Myofibrillar protein content. In a previous paper (Robinson, 1952) the composition of various protein fractions of chick embryonic muscle has been discussed. It has been concluded that a strong potassium chloride-phosphate extract will contain the majority of the myofibrillar and nuclear proteins of the muscle and that these will be precipitated together on dilution with addition of ethanol (Fraction 2). A small percentage will, however, remain behind in the residue and will be extracted from this, together with other proteins, by 0·1 N-sodium hydroxide (Fraction 3). The changes during development in the total protein content of Fraction 2 and of Fractions 2 + 3 are represented by curves *A* and *A′* respectively in Figs. 2 and 3 (derived from the data of Robinson, 1952).

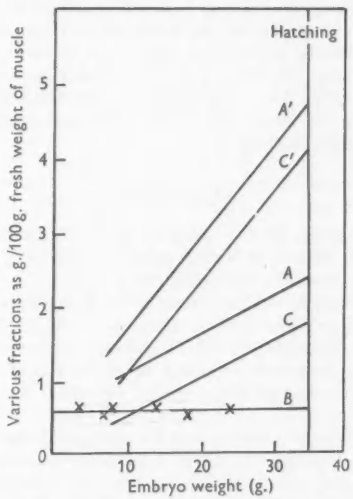

Fig. 2. Changes in the total protein, nucleoprotein and myofibrillar protein content of certain muscle fractions during embryonic development; expressed as g./100 g. fresh weight of muscle. *A*, total protein soluble in strong KCl-phosphate and precipitated by dilution and addition of ethanol (curve from data of Robinson, 1952). *A′*, total protein soluble in strong KCl-phosphate and precipitated by dilution and addition of ethanol plus that extracted from the residue by 0·1 N-NaOH (curve from data of Robinson, 1952). *B*, nucleoprotein content of the muscle residue after extraction with dilute salt solutions. *C*, resultant curves obtained by subtraction of the nucleoprotein contribution (curve *B*) from the total protein of curve *A*. *C′*, resultant curve obtained by subtraction of the nucleoprotein contribution (curve *B*) from the total protein of curve *A′*.

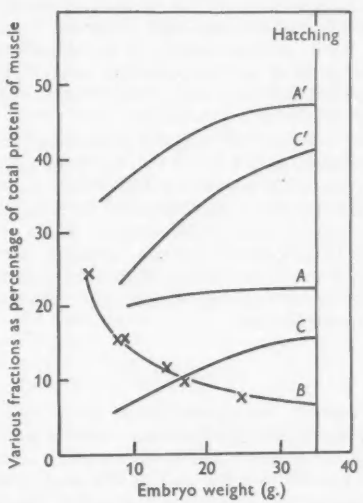

Fig. 3. Changes in the total protein, nucleoprotein and myofibrillar protein content of certain muscle fractions during embryonic development; expressed as a percentage of the total muscle protein. *A*, total protein soluble in strong KCl-phosphate and precipitated by dilution and addition of ethanol (curve from data of Robinson, 1952). *A′*, total protein soluble in strong KCl-phosphate and precipitated by dilution and addition of ethanol plus that extracted from the residue by 0·1 N-NaOH (curve from data of Robinson, 1952). *B*, nucleoprotein content of the muscle residue after extraction with dilute salt solutions. *C*, resultant curve obtained by subtraction of the nucleoprotein contribution (curve *B*) from the total protein of curve *A*. *C′*, resultant curve obtained by subtraction of the nucleoprotein contribution (curve *B*) from the total protein of curve *A′*.

By subtracting the nucleoprotein contribution to these fractions we can therefore estimate the true myofibrillar protein content. This has been done for the proteins soluble in strong potassium chloride-phosphate and precipitated by ethanol on dilution in curve *C* (Fraction 2), and for these proteins plus those extracted from the residue by 0·1N-sodium hydroxide in curve *C'* (Fraction 2+3).

The latter correction is the more justified since the whole of the deoxyribonucleoprotein is present in Fractions 2+3, whereas only 80% is present in Fraction 2 alone. However, because of the greater complexity of Fraction 3, which, particularly in the early embryonic stages, is likely to include material other than that derived from the myofibrils and the nuclei, this estimate of the amount of myofibillar proteins is probably too high. Fraction 2, on the other hand, consists solely of myofibrillar and nuclear proteins and the corrected curve *C*, therefore, should represent more closely the changes in the myofibrillar protein content.

After correction for the deoxyribonucleoprotein, the resultant curve for the true myofibrillar protein contribution falls away towards the beginning of the incubation period. Thus the large increase in the percentage of myofibrillar protein which occurs during development becomes apparent and the curves, *A* and *A'*, are seen to be the resultants of the decreasing nucleoprotein content and the increasing myofibrillar protein content.

DISCUSSION

It is clear that throughout the period of embryonic development the nucleoprotein content of the strong potassium chloride-phosphate extract of muscle is very high. It appears that at the earliest stages very little of the protein precipitated in Fraction 2 can be truly myofibrillar, if we acknowledge that the analyses of the nuclei are even approximately correct. Even at the 14-day incubation stage, when the average embryo weight is approximately 10 g., the nucleoprotein amounts to 66% of the total protein of this fibrillar fraction. In fact, the myofibrillar protein forms only 7% of the total protein of the muscle at this stage.

Despite the preponderance of the nucleoproteins in these early stages, the breast muscle will respond to electrical stimulation after incubation for 8 days. The exact relation of such a contraction to the organized activity of the adult myofibril is not clear but it appears that, regardless of the great differences in the relative composition, the same contractile organization is present and functional.

Hermann & Nicholas (1949) studied the nucleic acid distribution in developing rat muscle. Under their conditions of fractionation the majority of the DNA remains unextracted, and they claim that it does not interfere with their estimations of the fibrillar proteins. However, it is likely that a considerable proportion of the myofibrillar proteins themselves must similarly remain unextracted under their conditions. It is difficult to draw comparisons between the two analyses; not only do the experimental procedures differ, but species differences may also be significant. Nevertheless, the total nucleic acid content and the DNA/RNA ratio seem to be of the same order at corresponding developmental stages.

SUMMARY

1. An assessment has been made of the nucleoprotein content of certain protein fractions of chick breast muscle in the prehatching period.

2. Nucleic acid analyses of these fractions, and nucleic acid and protein analyses of isolated muscle nuclei, have been made. From these determinations the nucleoprotein content of the fraction which contains the proteins of the myofibril has been calculated.

3. A better measure of the changes in the myofibrillar protein content has been obtained by correcting previous analyses for the nucleoprotein contribution. The large increase which occurs during embryonic development in the percentage of myofibrillar protein is thus made apparent.

4. The low myofibrillar and high nuclear protein content of muscles which, nevertheless, respond to electrical stimulation has been commented upon.

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A Study of the Adenosinetriphosphatase Activity of Developing Chick Muscle

By D. S. ROBINSON
Biochemical Laboratory, University of Cambridge

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In 1939, Engelhardt & Lyubimova (1939) first made the important observation that a close association exists between the enzyme adenosinetriphosphatase (ATP-ase) and the myofibrillar protein, myosin, and they tentatively suggested that the enzyme might in fact form part of the myosin molecule itself. Since that time their observations have been amply confirmed by other workers. Moreover, the significance of the connecting link which is thus provided between the chemical events during muscle contraction and the protein systems presumed responsible for the mechanical changes has been widely appreciated. The muscle ATP-ase studied by Kielley & Meyerhof (1948, 1950) appears to be a distinct enzyme present in water extracts, and associated with the cell particulate bodies rather than with the myofibrils (Perry, 1952).

A study of the muscle ATP-ase during the early stages of development of the chick was begun in conjunction with other work on the developing tissue (Robinson, 1952*a*, *b*). The homogenized muscle was extracted with 0·6M-potassium chloride in 0·067M-borate buffer at pH 7. A sample of the extract was diluted to 0·05M-potassium chloride, when the myofibrillar proteins precipitated, and could be centrifuged down. The enzyme activity was determined in the whole extract, in the precipitate on dilution and in the residue left after extraction with the 0·6M-potassium chloride solution. The presence of a high proportion of the total ATP-ase activity in water extracts from muscle early in embryonic development led to a further more detailed study of such extracts.

EXPERIMENTAL

Most tissues possess a variety of enzymes whose action results in the liberation of inorganic phosphate. Thus, alkaline and acid phosphatases, pyrophosphatases and myokinase might interfere with any attempt to estimate a specific ATP-ase activity, and it is necessary to try to define conditions for an ATP-ase estimation which will minimize any interference by such systems.

In view of the importance of the myosin ATP-ase system, many workers have investigated its properties. Not all the results are in agreement, particularly with respect to the pH optimum and the effect of ions upon the activity. However, Mommaerts & Seraidarian (1947) have been able to account for most of the differences in terms of the extreme sensitivity of the myosin ATP-ase towards electrolytes, and to show that, provided the ionic conditions are strictly controlled, it is possible to develop procedures for the specific assay of myosin ATP-ase. It is activated by calcium ions at low potassium chloride concentrations and has its maximum activity around pH 9, though the behaviour is in general too complex to be described in terms of a constant pH optimum. Myokinase will have little effect on the initial velocity if this is measured in the presence of excess substrate; moreover, the specificity of a preparation and its freedom from alkaline and acid phosphatases and from pyrophosphatases can readily be tested.

It is more difficult to define conditions under which the Kielley & Meyerhof (1948, 1950) ATP-ase of muscle will not be estimated. This enzyme is, however, relatively unstable in the absence of cyanide ions and has a maximum activity in the presence of magnesium ions at pH 6·8: the test conditions to be described are such as to minimize its contribution to the activity measurements.

Preparation of adenosinetriphosphate. The barium salt of adenosinetriphosphate (ATP) was prepared by a method similar to that described by Needham (1942) by Mr E. J. Morgan. It was converted to the sodium salt as recommended by Bailey (1942). The salt solution was freed from

iron by shaking with 8-hydroxyquinoline in $CHCl_3$ at pH 7 until no coloration was given by the 2:2′-dipyridyl test for iron. The solution was then shaken with pure $CHCl_3$ to remove 8-hydroxyquinoline and finally the $CHCl_3$ was removed *in vacuo*. The free inorganic phosphate, estimated by the Fiske & Subbarow (1925) method, was negligible in these preparations. ATP concentrations are expressed in terms of acid-labile phosphorus, which forms two-thirds of the total phosphorus, and hence twice the amount liberated by myosin ATP-ase. It is estimated by hydrolysing at 100° for 10 min. in N-HCl under which conditions both pyrophosphate linkages are broken (Bailey, 1949).

Preparation of adult fowl myosin ATP-ase. Muscle dissected from the breast was finely cut with scissors in an extractant having the following composition: 0·6 M-KCl, 0·067 M-borate, pH 7·4. After homogenization in the Waring Blendor for 30 sec. the suspension was stirred for 15 min. and finally centrifuged for 30 min. at 1200 *g*. The supernatant fluid was diluted to 0·05 M-KCl and the precipitate centrifuged down. Further purification was achieved by dissolving the precipitate in 0·8 M-KCl and, after centrifuging off any insoluble material, precipitating by dilution to a molarity of 0·05 M. The preparations were twice reprecipitated in this way so as to be practically free of any myokinase activity. Glass-distilled water was used throughout and the temperature was maintained at 0°.

Preparation of fractions from embryonic muscle. The extracting solution had the same composition as in the preparation of the adult ATP-ase. From 4 to 8 g. of breast muscle were harvested and a muscle extract prepared as before in 0·6 M-KCl-borate.

A sample of the extract was diluted to 0·05 M-KCl and the precipitate spun down on the centrifuge and retained. This myofibrillar fraction was expected to contain most of the ATP-ase activity and was not further purified. The whole extract of embryonic muscle, the precipitate on dilution of the extract and the residue left after extraction were tested for their ATP-ase activity. The total enzyme content of the residue was never more than 5 % of that of the myofibrillar fraction.

Preparation of aqueous extracts of embryonic muscle. Muscle dissected from the breast was homogenized in the Waring Blendor with an equal volume of glass-distilled water for 30 sec. and the suspension stirred for 15 min. at 0°. After centrifugation for 30 min. at 1200 *g*, second and third extractions were carried out and the supernatants combined at neutral pH.

Conditions of enzyme tests. These were developed on the basis of an examination of the ATP-ase associated with the adult myofibril. It was assumed that the enzyme present in the developing tissue would behave similarly, and consequently the test conditions were such as to be optimal for the adult ATP-ase. However, in order to increase the specificity of the test, the activity was measured at two pH values, 8·8 and 6·6. If the ratio of activities at these two values remains constant it provides additional evidence that the same enzyme system is present throughout in all the fractions.

The incubation system recommended by Bailey (1942) was used. The final concentrations in all the tests were as follows: ATP, 500–600 μg. acid-labile P/ml.; $CaCl_2$, 0·02 M; KCl, 0·1 M; buffer solution, 0·066 M. Glyoxaline (buffering range: pH 6·2–7·8), 4-methylglyoxaline hydrochloride (buffering range: pH 7·0–8·25) and 2:4-dimethylglyoxaline hydrochloride (buffering range: pH 8·0–9·3) were used as buffers covering the whole range pH 6·2–9·3. The tubes were incubated at 25° for periods of 1, 2, 3, 4 and 5 min. at two pH values, 8·8 and 6·6, and the reaction arrested by adding trichloroacetic acid to a final concentration of 5 % (w/v).

Under the above conditions excess ATP was present throughout, and there was no falling off in the rate of liberation of inorganic phosphate. This method of activity determination seems preferable to the single reading generally used, since any decrease in activity with time is immediately apparent.

RESULTS

Preliminary investigations of the activity of adult fowl myosin adenosinetriphosphatase

The behaviour of adult fowl myosin ATP-ase appears to be directly akin to that associated with rabbit myosin. The pH-activity curve shows two peaks, at pH values of 8·8 and 6·6, in glyoxaline buffers containing 0·1 M-potassium chloride and 0·02 M-calcium chloride at 25° (Fig. 1). The enzyme is

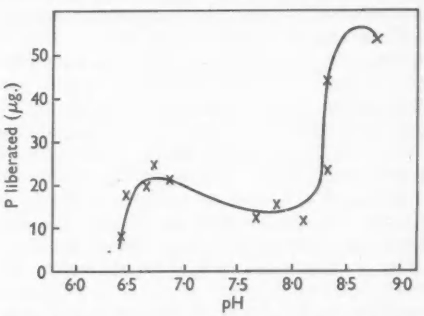

Fig. 1. pH-Activity curve of adult fowl myosin ATP-ase at 25° in glyoxaline buffers containing 0·1 M-KCl and 0·02 M-$CaCl_2$.

relatively unstable at high pH values and with periods of 10 min. incubation considerable inactivation occurs even at pH 8·5. The varied values for a pH optimum reported in the literature (Banga, 1942; Laki, 1943; Biro & Szent-Györgyi, 1949) may be due to such partial inactivation and it seems essential that only initial velocities should be used in the determination of the myosin ATP-ase pH curve.

In the presence of low concentrations of potassium chloride the enzyme shows marked calcium activation and very slight magnesium activation, in general agreement with previously published observations on rabbit ATP-ase (Bailey, 1942). In a typical experiment in 0·1 M-potassium chloride at pH 8·8, 15 μg. P were liberated in the absence of calcium and magnesium ions, 18 μg. P in the presence of 0·02 M-magnesium chloride and 47 μg. P in the presence of 0·02 M-calcium chloride, in 2 min. at 25°. Glyoxaline buffers have consistently given the highest activity.

The adenosinetriphosphatase activity of developing muscle

The results will be presented (*a*) in terms of the activity of the proteins associated with the myofibril; that is, the sum of the activities of the precipitate obtained on dilution of the 0·6M-potassium chloride extract and of the residue left after the extraction; and (*b*) in terms of the activity of the sarcoplasmic proteins not precipitated on dilution, the latter being calculated from the difference between the activities of the whole extract and the myofibrillar precipitate.

studied almost all of the ATP-ase remains in the supernatant solution on dilution of strong salt extracts of the muscle. As growth continues there is a progressive decrease in the proportion of ATP-ase in this fraction and a corresponding increase in the proportion associated with the precipitated myofibrillar proteins. Even at hatching, however, only 65% of the total ATP-ase, as measured under the specified test conditions, is associated with the myofibrillar protein precipitate.

When the activity of the myofibrillar fraction is expressed in terms of its protein content (using the data of Robinson, 1952*a*, *b*) it rises to a peak prior to

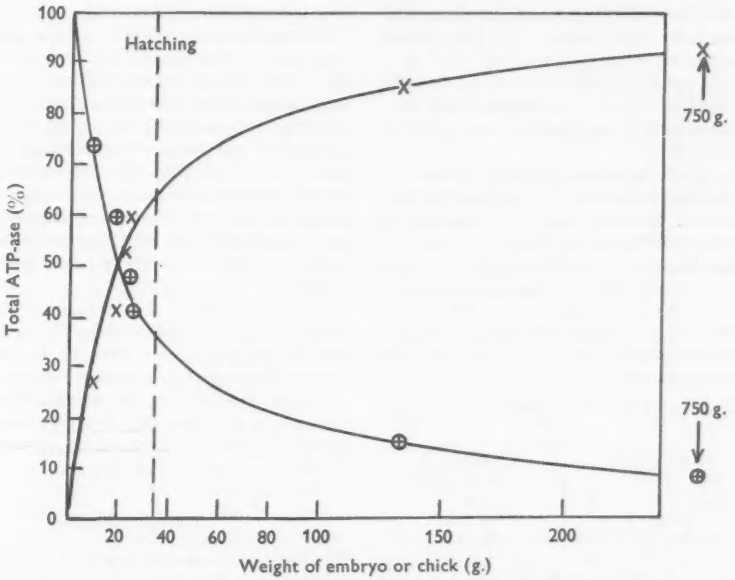

Fig. 2. The distribution of ATP-ase activity in the muscle of the developing chicken. ×, myofibrillar ATP-ase; ⊕, sarcoplasmic ATP-ase.

Table 1. *The adenosinetriphosphatase activity of the myofibrillar protein fraction of developing muscle*

Average weight of chick (g.)	Activity (mg. P/min./g. myofibrillar protein at 25°)*
10·3	36·7
14·7	38·6
23·3	55·8
25·3	53·3
Hatching	
134	34·0
750	35·0

* Corrected for the nucleoprotein contribution.

The percentage of the total ATP-ase possessed by each of these fractions at different periods is shown in Fig. 2. In the earliest developmental stages hatching (Table 1). A similar peak value was reported by Herrmann, Nicholas & Vosgian (1949) in studies on developing rat muscle.

The adenosinetriphosphatase activity of water extracts of embryonic chick muscle

The forms of the two curves in Fig. 2 would suggest at first sight that a process exists whereby the ATP-ase activity passes into the myofibrillar fraction and becomes closely associated with the fibril as development proceeds; in other words, that a water-soluble ATP-ase becomes associated with the myofibril and gives rise in the adult to an ATP-ase firmly combined with the myosin proper. However, no direct estimation of the ATP-ase in

water extracts had thus far been made, and a detailed examination of these was therefore undertaken.

pH-*Activity curve*. This was determined in the presence of 0·2M-potassium chloride and 0·001M-calcium chloride; it again shows two peaks, a large one in the alkaline region about pH 8·8–9·0 and a smaller peak around pH 6·6–7·0. Although the preparation is rich in myokinase, as almost 100% of the total acid-labile phosphorus is released on long incubation at neutral pH, this activity should have no effect on the pH curve which is based on measurements of the initial velocities.

Specificity of the preparation. The activity of the preparation towards inorganic pyrophosphate and β-glycerophosphate was tested. Pyrophosphate was not attacked either at pH 8·8 or at pH 7·0, at which latter pH pyrophosphatase activity is usually maximal. At pH 8·8 the activity towards β-glycerophosphate was very slight amounting to only 5% of that towards ATP.

Influence of ions. The activity in the presence of different ions suggested a closer relationship to the adult myofibrillar ATP-ase than to the Kielley & Meyerhof (1948, 1950) enzyme (Table 2), since the activity is raised markedly in the presence of calcium ions but little in the presence of magnesium ions.

Table 2. *The influence of ions upon the activity of the adenosinetriphosphatase present in water extracts of chick embryo muscle*

(Activity measured at pH 8·8 in 2:4-dimethylglyoxaline buffer.)

Ions present in addition to K^+ and Cl^-	Activity (μg. P released/3 min.)
No addition	21·5
Mg^{++} (0·02M)	29·0
Ca^{++} (0·02M)	76·0

Miscellaneous observations. When stored at pH 7 at 0° the enzyme loses activity only at a slow rate, but is much less stable at lower pH values. Thus at pH 5, 80% of the activity is lost in 5 min. at 0°. Fractionation with ammonium sulphate shows that the enzyme is precipitated over a wide range between 20 and 50% saturation. Some of the enzyme activity is lost after precipitation with ammonium sulphate and a further diminution occurs on dialysis. Using the unit of activity, *Qp*, defined by Lyubimova & Engelhardt (1939) as the volume of a hypothetical gas (μl.) equivalent to the amount of P (in μg.) liberated by 1 mg. of myosin in 1 hr., freshly prepared water extracts give values as high as 1500 units at 25°. These can be compared with average values for freshly prepared fowl myosin ATP-ase which range from 1300 to 1500 at 25° and are similar to those obtained by Bailey (1942) for chick myosin at 37°. Extraction of the tissue with 0·1M-potassium chloride instead of water gave an extract of comparable activity.

In all these respects the activity of water extracts shows a closer resemblance to the adult myosin ATP-ase than to the Kielley & Meyerhof (1948, 1950) system, though the latter is associated with the cell granules which would also aggregate and come out of solution at similar ammonium sulphate concentrations.

High-speed centrifugation of water extracts

Centrifugation of a water extract of embryonic muscle at 13 000 *g* for 60 min. left some 60% of the ATP-ase in the supernatant while about 40% appeared to be associated with the precipitate.

Some preliminary electron microscope studies of this precipitate, made in collaboration with Mr R. W. Horne of the Cavendish Laboratory, Cambridge, showed that in addition to the cell granules, it contained fragments of elementary myofibrillar material. Such material has been studied in some detail in preparations from the whole embryonic muscle. Although fibrillar in nature it appears to be very fragile and tenuous, and has a much simpler banding system than can be observed in similar preparations from the adult muscle.

Examination with the electron microscope of the residue remaining after water extraction of embryonic muscle shows that this fraction contains similar rudimentary myofibrils and, in addition, considerable amounts of reticular material, the presence of which was anticipated from histological knowledge of the composition of the embryonic tissue (Robinson, 1952*a*, Fig. 8).

DISCUSSION

The present studies have shown that in the earlier developmental stages of the chick only a small proportion of the total muscle ATP-ase is present in the precipitate formed on dilution of strong salt extracts of muscle, most of the activity remaining in the supernatant solution. With further development a progressive change in the distribution occurs until in the adult practically the whole of the ATP-ase activity is associated with the myofibrillar precipitate.

In previous work on the protein distribution in embryonic muscle (Robinson, 1952*a*) low concentrations of ethanol have been used to complete the precipitation of the myofibrillar proteins on dilution of a strong salt extract of the muscle. Because of this difference in procedure no direct comparisons can be made between the protein distribution found then and the ATP-ase distribution studied here, since in the embryonic extracts ethanol may have caused the precipitation of the

sarcoplasmic ATP-ase and its estimation with the myofibrillar proteins.

In view of the activity of the Kielley & Meyerhof (1948, 1950) ATP-ase in adult muscle, it is necessary to examine more closely the estimation procedures which have been used. It seems clear from the well defined calcium activation and optimum activity around pH 8·8 shown by all the enzyme preparations from embryonic muscle, that the main enzyme studied is very similar to that first described by Engelhardt & Lyubimova (1939). The Kielley & Meyerhof ATP-ase is magnesium-activated and has an optimal activity around pH 6·8. On these grounds our observations support the view that in our studies on the embryonic tissue we have been mainly concerned with the Engelhardt-Lyubimova enzyme. It seemed likely that the Kielley & Meyerhof ATP-ase might have been active in that fraction precipitated on high-speed centrifugation of water extracts. However, detailed studies of the activity of this fraction show that in the embryonic preparations the calcium-activated enzyme is responsible for most of the activity even at pH 6·6. As anticipated, in similar preparations from the adult, the magnesium-activated system prevails. The author is grateful to Dr S. V. Perry (1952) for information concerning the tests he has elaborated to distinguish between these two enzymes.

Herrmann & Nicholas (1948) studied the Ca^{++}-activated apyrase activity of developing rat muscle and found that after the 16th day of incubation the total activity, expressed in terms of the dry weight of the muscle, begins to rise from a low level and about 3 weeks after birth reaches an approximately constant value. Later studies (Herrmann *et al.* 1949) of particular protein fractions, showed that at the earlier developmental stages only a small amount of the total apyrase activity was contributed by the myofibrillar fraction, in general agreement with our results.

The finding that water extracts of embryonic muscle contain an adenosinetriphosphatase which is apparently closely related to that associated with myosin in the adult, is particularly striking in view of the evidence for the possible identity of myosin and that enzyme (Bailey, 1942; Bailey & Perry, 1947; Szent-Györgyi, 1947). At present, however, it does not seem possible to decide whether we are concerned with a true separation of myosin and its ATP-ase activity, or whether the results are explicable in terms of the elaboration of the myofibrillar elements of muscle without assuming such a separation of protein and enzyme.

An interpretation in the latter sense is certainly possible. For instance, a water-soluble prototype of the myosin molecule possessing ATP-ase activity can be envisaged, or possibly the elaboration of the adult myofibril may proceed by some form of aggregation of elementary protofibrillar structures which in the earlier embryonic stages might be present in water extracts. The electron microscope work described may be quoted in support of such a view. Certainly such concepts would account for the ATP-ase distribution with which we have been concerned without the necessity of questioning the identity of myosin and ATP-ase.

SUMMARY

1. The distribution of adenosinetriphosphatase activity has been studied in the developing chick embryo.

2. Muscle extracts containing the sarcoplasmic and myofibrillar proteins have been prepared in 0·6M-potassium chloride-borate solution and the myofibrillar proteins precipitated on dilution to 0·05M-potassium chloride. In the adult most of the muscle adenosinetriphosphatase is present in the precipitate in association with the protein, myosin.

3. The adenosinetriphosphatase activity of the whole extract and of the precipitate has been determined at different developmental stages. In the earliest embryos studied the percentage of the total activity in the sarcoplasmic fraction is high in comparison with that in the myofibrillar protein fraction alone. As development proceeds the distribution changes and the enzyme becomes more closely associated with the myofibril.

4. The adenosinetriphosphatase activity of the sarcoplasmic fraction has been studied in water extracts of embryonic muscle. Its relation to the adult myosin adenosinetriphosphatase and to that associated with the cell particulate systems of muscle has been considered. The work cannot be related to previous studies on the protein distribution in chick muscle since the methods of preparation of the fractions are different. Some possible interpretations of the results are discussed.

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The Amino-Acids and other Ampholytes of Urine

1. A GENERAL METHOD OF ISOLATION

By R. G. WESTALL
Medical Unit, University College Hospital Medical School, London, W.C. 1

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The nitrogenous constitutents of urine have been studied by many workers since the earliest days of biochemistry. Various methods have been devised for the estimation of the amino-acids and other nitrogenous components, but the actual isolation of the large variety of substances present in relatively small amounts has proved to be tedious and time-consuming. The invention of paper chromatography and the production of the newer synthetic ion-exchange resins has now provided a means whereby further studies of the nitrogenous substances which occur in urine have become more practicable. New interest in such methods has been stimulated by the discovery that in the routine examination of normal and pathological urines by paper chromatography carried out by Dent (1947, 1948) and his co-workers during the past 5 years, ninhydrin-reacting spots have been observed which could not be ascribed to any of the known naturally occurring amino-acids. Moreover, in almost every case, the positions of the unknown spots on the paper chromatograms were unchanged after hydrolysis by acid and this indicated that the unknown constituents were probably amino-acids and not peptides. Several of these substances have now been isolated from urine and preliminary communications have been published (Crumpler, Dent, Harris & Westall, 1951; Dent, Fowler & Walshe, 1951; Searle & Westall, 1951). The early stages of the method used for the isolation of these amino-acids were essentially the same in each case, and in order to avoid unnecessary repetition the general method of separation will be described in this paper, whilst the details of the isolation of the individual substances will be given in later papers of this series.

The procedure employed has evolved from studies on displacement chromatography on the synthetic ion-exchange resins Zeo-Karb 215 and De-Acidite E (Partridge & Westall, 1949; Partridge & Brimley, 1949). The fractionation of the amino-acids of acid-hydrolysed proteins of egg white has been described (Partridge, 1949) and further application has led to the isolation of γ-aminobutyric acid from beetroots (Westall, 1950*a*) and for the preparation in quantity of glutamine from the same source (Westall, 1950*b*). The application of the method, now modified by using the anion-exchange resin Dowex 2 instead of De-Acidite E, to the isolation of amino-acids from urine has not, so far, been described in detail.

General principles

The direct application of diluted urine to the Zeo-Karb columns resulted in the production of a gel within the column which disturbed the even flow of the liquid. This was due partly to traces of protein but also to some other ultrafilterable substance.

This disturbance is eliminated by evaporating the urine to small volume under reduced pressure and then adding 3 vol. of ethanol. The precipitate which forms is removed by filtration and washed with 80% (v/v) aqueous ethanol. No loss of amino-acids is incurred and a proportion of the inorganic salts is removed, together with the interfering substances. The ethanol in the filtrate is removed by evaporation under reduced pressure and the resultant syrup is diluted with water to twice the volume of the original urine. The passage of this solution through the system of Zeo-Karb columns enables the inorganic acids, organic acids, sugars and all substances including urea not functioning as cations, to be eliminated, since they are all carried away in the effluent. Certain organic ampholytes which carry a strongly acidic group, e.g. taurine, are not retained. Ca^{2+}, K^+ and Na^+ are retained at the top of the first column with the concomitant release of an equivalent number of H^+. This causes the aqueous phase within the columns to become strongly acidic and under these conditions the capacity of the resin for the retention of cations is low (Partridge & Westall, 1949). Obviously the initial precipitation with ethanol only partially removes the inorganic salts, and the size of the columns used in the experiment has to be unduly large for the collection of the relatively small amounts of amino-acids present. However, after the primary separation, subsequent fractionations can be carried out on much smaller columns since, in the absence of an excess of H^+, the organic bases and ampholytes are retained in much larger amounts—equivalent to 1·5–2·5 m-equiv./g. dry resin.

Subsequent treatment of the Zeo-Karb columns with 0·2N-ammonia displaces only those bases which are weaker than ammonia; the metal cations and some strong organic bases, as well as the basic amino-acids arginine and lysine, remain on the resin column. The effluent is allowed to run to waste until a positive ninhydrin colour is obtained. At this point, fractions are collected until the effluent is strongly alkaline. The amino-acid composition of the successive fractions is determined by paper chromatography (Consden, Gordon & Martin, 1944), whilst creatinine is identified by the Jaffé reaction as applied to paper chromatography (Maw, 1947). The degree of separation of the various ampholytes which is obtained by this primary fractionation is small owing to the large size of the columns. However, where interest is focused on one particular component, all the fractions which do not contain this substance may be discarded.

The secondary fractionation of the selected samples is carried out on a column system containing the anion-exchange resin Dowex 2. Some details on the use of this resin for the separation of amino-acid mixtures have been published (Partridge & Brimley, 1951*b*). The order in which the various amino-acids are displaced from this resin will be discussed later, but it can be stated here that the order is not simply the reverse of that obtained on the cation-exchange column. The ampholytes retained on the Dowex 2 columns are displaced with 0·1N-hydrochloric acid and again fractions are collected until the hydrochloric acid reaches the effluent. The fractions are analysed by means of paper chromatography as already described.

EXPERIMENTAL

Materials

The cation-exchange resin Zeo-Karb 215 (Permutit Company, London) was ground in a hammer mill and graded by sieving in the dried condition. The 40–60 mesh/in. grade was used for the large columns and 80–100 mesh/in. grade for small-scale use. The resin was allowed to stand overnight in 5N-HCl and, after several washes by decantation using distilled water, the columns were packed by sedimentation. The packed columns were treated once with N-NH_3 followed by water and then with 2N-HCl followed by water again until the pH of the effluent was 5. This alternate treatment with N-NH_3 and 2N-HCl served to consolidate the resin bed.

The anion-exchange resin Dowex 2 (Dow Chemical Company, Midland, Michigan), which is available in two grades, 30–40 and 250–500 mesh/in., and is supplied in the chloride form, was made up into a slurry with N-HCl and run into the columns. After allowing the resin to settle, it was washed with water and regenerated by applying 2N-NaOH (CO_2-free) followed by CO_2-free distilled water until the pH of the effluent fell below 8. Details of the precautions necessary to keep the system free from CO_2 have been reported (Davies, Hughes & Partridge, 1950).

Primary fractionation

In order to illustrate the method, a typical fractionation of human urine is described. The urine was collected from two subjects, both of whom excreted a proportion of β-amino-*iso*butyric acid which was high in comparison with the other amino-acids (Crumpler *et al.* 1951). The urine (18 l.) was evaporated to 2 l. under reduced pressure using a circulating evaporator (Van Heyningen, 1949), 3 vol. of ethanol were added and the mixture was allowed to stand overnight at 0°. After filtration, the solids were washed with 80% aqueous ethanol and the filtrate and washings were evaporated to remove the ethanol. The syrup was taken up with 3 l. of water and shaken with 100 g. of charcoal which had been previously treated with dilute acetic acid (Partridge, 1949). This procedure removed a large proportion of the tyrosine which tends to crystallize in the columns. Some phenylalanine is also retained on the charcoal. The clear filtrate obtained after removal of the charcoal was diluted to 36 l. and allowed to run on to the system of Zeo-Karb columns at a rate not exceeding 1 l./hr. The set of columns, already described (Partridge, 1950), was made of three sections placed side by side and connected up in series with narrow glass tubing. The first section (diam. 3 in., height 30 in.) contained 1100 g. (dry wt.) of Zeo-Karb 215 (40–60 mesh/in.); the second section (diam. 2 in., height 24 in.) contained

330 g. resin; and the final section (diam. 1·5 in., height 15 in.) contained 130 g. of resin. During the application of the solution, and also whilst a further 5 l. of distilled water was applied to replace the acid remaining in the columns, the effluent was allowed to run to waste.

The amino-acids were displaced by 0·2 N-NH_3 which was applied at a rate of 1 l./hr. Again the effluent was discarded until a positive ninhydrin reaction was obtained. At this point, fractions (50 ml.) were collected. Thirty-five fractions were taken including three collected beyond the point at which the dark NH_3 band reached the end of the last column. The amino-acid composition of the successive fractions was determined by placing 10 μl. samples at 2 cm. intervals along the base-line of sheets of no. 4 Whatman filter paper. One set was run with phenol-NH_3 and a duplicate set with collidine-lutidine mixture. After drying, the sheets were developed with ninhydrin in the usual way. Further sheets were prepared, run in the same solvents, and developed with alkaline picric acid solution to test the presence of creatinine.

with resin ground to about 60 mesh/in.; the third section (diam. 0·75 in., height 3 in.) again contained resin of 60 mesh/in.; whilst the final section (diam. 0·35 in. height 2·25 in.) was filled with resin of 250–500 mesh/in. The dimensions given are those for the internal diameter of the tubes and the height is the actual length of the column of resin. The tubes were, of course, longer to allow for head-space. It will be noticed that these columns are relatively shorter and wider than those used for the Zeo-Karb resin. This is due to the fact that the Dowex resin swells when the amino-acids solutions are applied and the use of long narrow columns would lead to the production of a considerable internal pressure. The fourth section, which restricts the flow rate through the system, was only placed in position just before the displaced amino-acids emerged from the third column, as shown by the swelling of the resin. The effluent, which contained creatinine, together with some creatine, was collected for investigation later. The columns were washed with 1 l. of distilled water after all of the

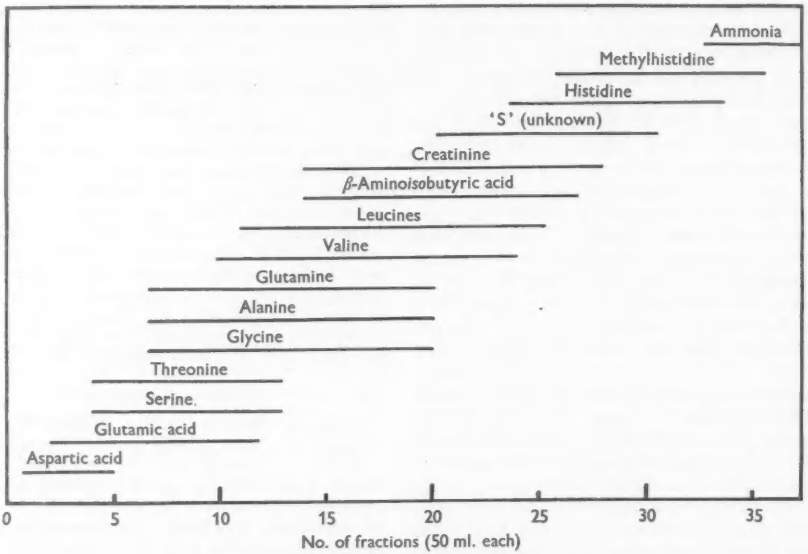

Fig. 1. Primary fractionation on Zeo-Karb 215.

Secondary fractionation

The first nine fractions from the previous separation which contained aspartic acid, glutamic acid, glycine and alanine, together with several other substances present in traces, were set aside. The remaining fractions (10–35), which contained the amino-acid selected for isolation, were bulked, heated to 60° under reduced pressure to remove CO_2 and applied to a system of columns containing the anion-exchange resin Dowex 2. This set was made up of four columns of decreasing size connected in series with narrow glass tubing. The first column (diam. 2 in., height 8 in.) contained the coarser grade of resin (approx. 30 mesh/in.); the second column (diam. 1·25 in., height 4·5 in.) was filled

amino-acid solution had run on. The amino-acids were now displaced by applying 0·1 N-HCl and fractions (25 ml.) were collected as soon as a positive ninhydrin reaction was obtained with the effluent. The collection was continued for two fractions beyond the point at which a positive chloride test was obtained. The amino-acid composition of the fractions was determined chromatographically in the manner already described.

RESULTS

The composition of the samples contained from the primary fractionation is shown in Fig. 1. It has been pointed out that considerable overlapping of the

amino-acid bands must be expected under the conditions imposed by the large quantity of inorganic salts present in the urine. A repeated fractionation using smaller columns, a reduced rate of flow during development and the collection of smaller and more numerous fractions will give a far greater resolution of the mixture. However, it is the writer's belief that it is preferable to proceed directly to a secondary fractionation on Dowex 2 and thus eliminate creatinine from the mixture. Then, if necessary, a further fractionation may be carried out on Zeo-Karb 215 of those selected fractions which contain the particular amino-acid which it is intended to isolate. Only those components which occur in relatively large amounts are indicated in Fig. 1. The composition of the fractions obtained from the

DISCUSSION

The method, so far outlined, is intended to provide an initial treatment whereby the various amino-acids and other ampholytes including simple peptides can be concentrated in a number of fractions. The actual isolation in pure form of any one component from this comparatively simple mixture will usually call for the use of additional methods, but the isolation of a substance occurring in large amounts relative to other amino-acids can often be achieved by ion-exchange fractionation alone. If, however, several amino-acids form mixed bands on both resins it may be possible to obtain a separation by using the Zeo-Karb column at a higher temperature and thus minimize the non-ionic

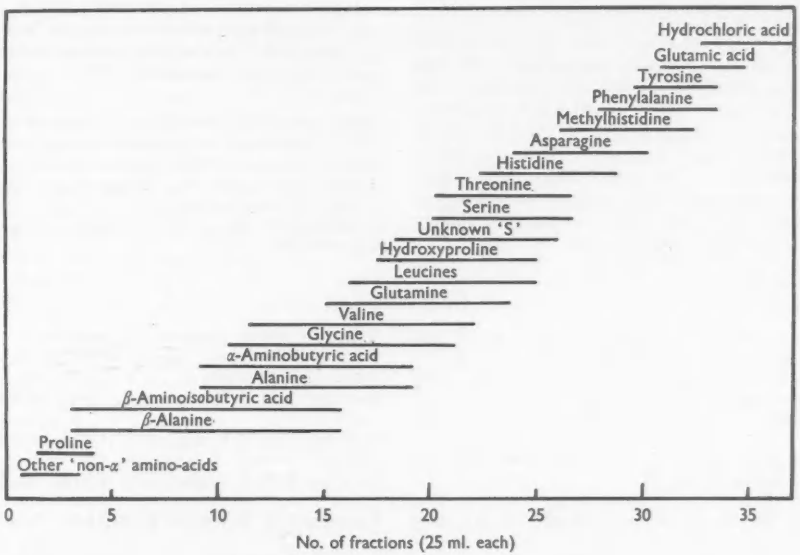

Fig. 2. Secondary fractionation on Dowex 2.

fractionation on Dowex 2 is shown in Fig. 2. The various amino-acids listed cannot all be recognized readily from one-way paper chromatograms, and it is necessary to carry out two-way chromatograms on doubtful fractions in order to obtain unambiguous identification. Also, with the exception of '*S*' substance, an unknown, the amino-acids named in the figure are limited to those which are the main constituents of urine (Dent, 1948). Consideration of other substances occurring in small quantities in these fractions will be reserved for a later paper.

absorption effect. Useful separations have been achieved in this way (Partridge & Brimley, 1951*a*). If the mixture is not resolved under these conditions it is unlikely that any separation will be gained by any method which depends on the electrical properties of the charged ampholytes, and other properties such as specific absorption, partition coefficients between aqueous and organic solvents or even purely chemical methods may have to be used.

Partridge & Brimley (1951*b*), in their paper on the behaviour of amino-acids on Dowex 2, indicated

the order in which these substances are displaced from the resin. This order is consistent with the theory (Davies, 1949) which assumes that the order of emergence is determined by the relative values of pK_2 of the amino-acids. Reference to the table of apparent dissociation constants of amino-acids (Cohn & Edsall, 1943) shows that apart from arginine and lysine, both of which are absent from the Dowex fractionation, those amino-acids in which the amino group is further removed from the carboxyl group than in α-amino-acids have values for pK_2 which are higher than those of α-amino-acids except proline and hydroxylysine. Consequently, it would be expected that these amino-acids would be the first to emerge from the Dowex 2 column, and reference to Fig. 2 shows that this is so. In fact it may be taken that any amino-acid, with the exception of proline and hydroxylysine, which is displaced from a Dowex 2 column under the conditions described in front of the alanine band is most probably a 'non-α' amino-acid. In the experiment described, the first three fractions from the Dowex column have yielded small amounts of several amino-acids in addition to β-alanine and β-amino*iso*butyric acid and proline. These unknown substances survive as visible spots on a paper chromatogram treated with basic copper carbonate (Crumpler & Dent, 1949). This is additional evidence that they are 'non-α' amino-acids.

SUMMARY

1. A procedure is described whereby the amino-acids and other ampholytes may be separated from other urine constituents and to some extent from each other.

2. The primary separation is carried out on columns of the cation-exchange resin Zeo-Karb 215. Selected fractions from the initial separation are then refractionated on columns of the anion-exchange resin Dowex 2. In this way, the ampholytes, as a group, are concentrated in a number of arbitary fractions and a large degree of resolution of the complex mixture is also obtained.

3. The selection of fractions in which any one amino-acid of special interest occurs provides a rich source for the final isolation of this substance.

4. The order in which the 'non-α' amino-acids are displaced from a column of the strongly basic resin Dowex 2 is discussed.

The author wishes to express his gratitude to Dr C. E. Dent for his interest and constant encouragement. He also wishes to thank the Dow Chemical Company for a gift of the Dowex 2 resin. The expense of this research was defrayed by a grant from the Rockefeller Foundation to the Medical Unit of the University College Hospital Medical School.

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The Paper-Chromatographic Examination of the Cardiac Aglycones of *Strophanthus* Seeds

By I. E. BUSH and D. A. H. TAYLOR
The National Institute for Medical Research, The Ridgeway, Mill Hill, London, N.W. 7

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The Medical Research Council Medicinal Plants Survey in its expedition to Africa collected many specimens of the seed of *Strophanthus hispidus* P.DC. and *S. sarmentosus* P.DC., mostly from individual plants, in order to investigate the occurrence of sarmentogenin, a substance which forms a possible starting material for the production of cortisone. The isolation of sarmentogenin from certain of these seeds has already been reported (Callow, Meikle & Taylor, 1951).

The present paper is a report on the preliminary investigation of all the samples collected, and reveals certain interesting features about the distribution of cardiac aglycones in various *Strophanthus* species.

As the examination of each seed sample by isolation of the constituents might take several years for completion, a method has been developed for the routine semi-micro investigation of the easily hydrolysable glycosides of the seeds. These easily hydrolysable glycosides (Reichstein, 1951), which are completely hydrolysed by refluxing for half an hour in 50% aqueous methanol which has been made 0·1N with respect to sulphuric acid, are those in which the steroidal portion of the molecule is directly attached in a glycosidic linkage to a 2-deoxy sugar. Most of the best known glycosides belong to this group, which is much easier to investigate than the group in which the aglycones are directly attached to 'normal' sugars carrying a hydroxyl group at $C_{(2)}$, such as glucose or rhamnose, where isolation of the intact aglycone is usually difficult and has in several cases not yet been achieved. Investigation of the 'normal' glycosides in our seed samples has so far only been carried out in selected cases.

The paper-chromatographic method of Zaffaroni, Burton & Keutmann (1950) (cf. Burton, Zaffaroni & Keutmann, 1951) has been used by Schindler & Reichstein (1951) for separating this type of substance, but the procedure is in many ways complicated

Table 1. *R_F values of some cardiac aglycones and derivatives in the system benzene/50% methanol at 37·5°*

(Systematic names follow the rules given in *J. chem. Soc.* 1951, p. 3526.)

Compound	R_F
'β'-Anhydrodigitoxigenin (3β-hydroxycarda-14:20(22)-dienolide)	0·91
'α'-Anhydrodigitoxigenin*	0·63
'β'-Anhydrodigoxigenin (3α:12β-dihydroxycarda-14:20(22)-dienolide)	0·73
'β'-Anhydrodigoxigenone (3:12-diketocarda-14:20(22)-dienolide)	0·59
'β'-Anhydrosarmentogenin (3β:11α-dihydroxycarda-14:20(22)-dienolide)	0·69
'β'-Anhydrouzarigenin (3β-hydroxy-5α-carda-14:20(22)-dienolide)	0·94
Digitoxigenin (3β:14-dihydroxycard-20(22)-enolide)	0·76
Digitoxigenin acetate	0·89
Digoxigenin (3α:12β:14-trihydroxycard-20(22)-enolide)	0·075
Digoxigenin diacetate	0·70
Digoxigenone (3:12-diketo-14-hydroxycard-20(22)-enolide)	0·68
Gitoxigenin† (3β:16ξ:14-trihydroxycard-20(22)-enolide)	0·26
Gitoxigenin diacetate	0·88
Periplogenin (3β:5:14-trihydroxycard-20(22)-enolide)	0·43
Sarmentogenin (3β:11α:14-trihydroxycard-20(22)-enolide)	0·10
Sarmentogenin diacetate	0·85
Sarnovide mono(?)acetate‡	0·72
Sarverogenin*	0·40
Strophanthidin (3β:5:14-trihydroxy-19-oxocard-20(22)-enolide)	0·16
Strophanthidol (3β:5:14:19-tetrahydroxycard-20(22)-enolide)	0·05
'Substance H'*	0·28
'Substance K'*	0·56
Uzarigenin (3β:14-dihydroxy-5α-card-20(22)-enolide)	0·83

* Compound of unknown structure.
† Reacts only weakly with the *m*-dinitrobenzene reagent.
‡ Callow & Taylor (1952).

by the treatment of the paper and the relatively long period of development required. The alternative systems developed to handle the steroid hormones (Bush, 1952) can easily be applied to the separation of cardiac aglycones, which are then detected by their colour reaction with 3:5-dinitrobenzoic acid and alkali. The advantage of these solvent systems is that no pretreatment of the paper is necessary; a continuous variation in the R_F values of a given set of compounds can be produced by slight variations in the solvent systems used; and the systems flow very quickly, allowing rapid and routine use of the method for analysing seed extracts or crystalline materials.

This method has been applied to a number of pure genins and to some simple derivatives still containing the $\alpha\beta$-unsaturated lactone ring, and the R_F values found in the system benzene/50% aqueous methanol are listed in Table 1. This system proved to be satisfactory for general use, although when only the more polar genins were present, the use of 60% methanol was found to give rather better separations. For substances which proved to flow at inconvenient rates in these systems modifications were made as described by Bush (1952).

In general it has been found that the R_F value decreases the larger the number of polar groups in the molecule, where these are widely separated; but where two or more polar groups occur close together, as in periplogenin, some form of vicinal action occurs which greatly increases the rate of flow.

The limitations of the method should be noted. Thus, whilst substances of only slightly different structure, such as digitoxigenin and uzarigenin which differ only in the configuration at $C_{(5)}$, can be separated quite adequately, other pairs of substances which differ widely in structure may have almost identical R_F values. An example of this is provided by 'β'-anhydrosarmentogenin and the partially acetylated sarnovide derivative (Callow & Taylor, 1952) which were for some time thought by us to be identical.

EXPERIMENTAL

A sample (1 g.) of the seed to be investigated was ground in a coffee mill and then extracted in a Soxhlet apparatus with methanol (150 ml.) for 2 hr. The extract was then evaporated to 25 ml., diluted with water (5 ml.) and extracted with light petroleum (3 × 25 ml.). The aqueous solution was then diluted with 0·1 N-H_2SO_4 (30 ml.) and refluxed for 30 min. After cooling, the solution was extracted twice with $CHCl_3$ (2 × 50 ml.) and the extract evaporated to 5 ml. One drop of this extract was sufficient for paper chromatography in the case of a seed with an average glycoside content. The chromatography itself was carried out as described by Bush (1952) in a system employing aqueous methanol as the stationary phase. For the routine analysis of large numbers of samples it was found convenient to employ an ascending chromatogram, although in this case the R_F values varied rather widely from one chromatogram to the next. The values given in Table 1 were obtained by employing a descending system, and are reproducible within the limits described by Bush provided that the appropriate precautions are taken.

For development of the spots a 1% solution of 3:5-dinitrobenzoic acid in 0·5 N 50% (v/v) aqueous methanolic KOH was found very suitable. This reagent gave a purple colour which had the advantage of being reasonably permanent, unlike the blue colour given by *m*-dinitrobenzene, which fades very quickly, in some cases even before the spots can be marked.

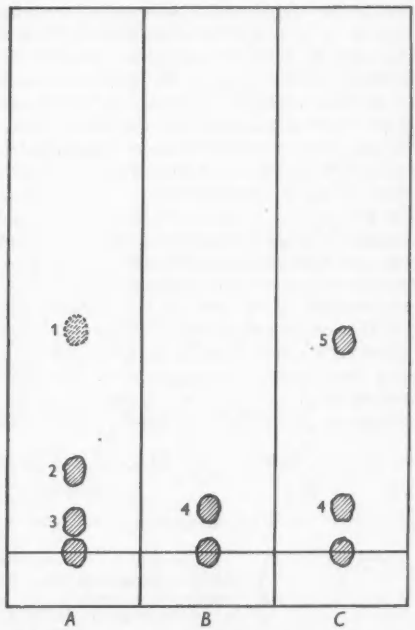


Fig. 1. Chromatogram given by typical seed extracts in an ascending benzene/60% methanol system. *A*, *S. hispidus*; *B*, *S. sarmentosus* ('savannah' form); *C*. *S. sarmentosus* ('forest' form). Spots: 1, periplogenin; 2, strophanthidin; 3, strophanthidol; 4, sarmentogenin; 5, sarverogenin.

RESULTS

The two species, *S. hispidus* and *S. sarmentosus*, have been found to give very different and readily recognizable chromatograms, differences between varieties of the same species being subordinate to the general pattern; typical chromatograms of each species are shown in Fig. 1.

The twenty samples of *S. hispidus* investigated have all been found to contain strophanthidin as their main constituent, together with a smaller amount of strophanthidol and a trace of peri-

plogenin, which has always been found to be present. Some specimens have also contained a fourth substance, 'substance *H*', which in one sample (CB/67; RDM/1032) gathered from a single tree at Kaboji near Kontagora, in Northern Nigeria, was present in as well. These are respectively the 'savannah' and 'forest' forms of Callow *et al.* (1951) and are, in the extreme cases, distinguishable botanically. The seed of the two forms has also a fairly characteristic appearance to the practised eye.

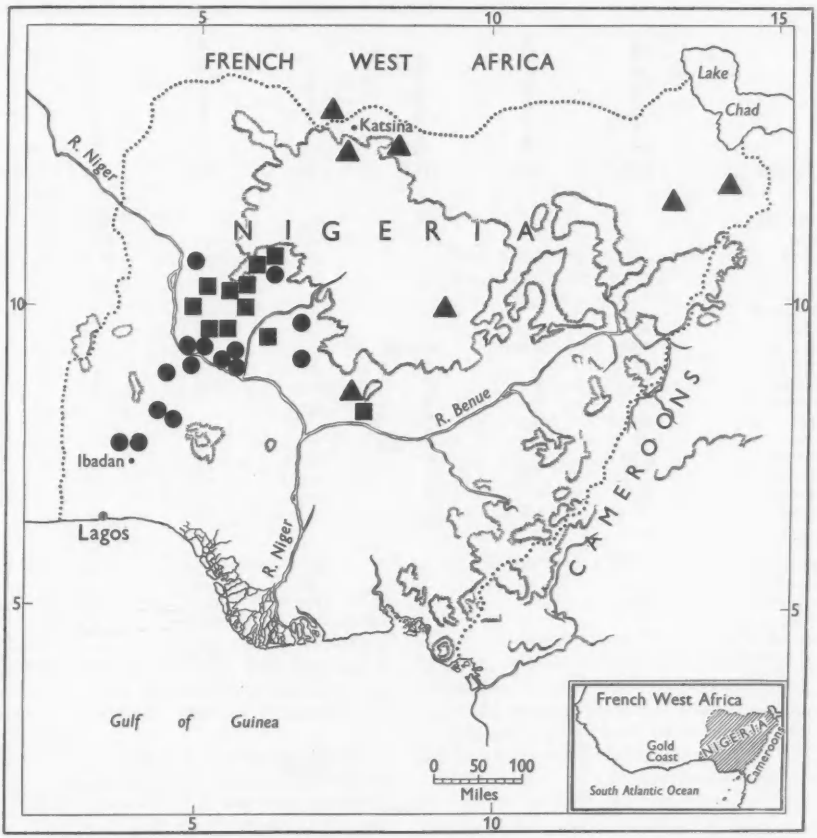


Fig. 2. Map showing places where seeds of *S. hispidus* and *S. sarmentosus* were collected. ■, indicates a plant of *S. hispidus*; ▲, a plant of the 'savannah' form of *S. sarmentosus*; and ●, a plant of the 'forest' type of this species.

roughly the same amount as strophanthidol. Another plant (CB/68) in the same village was of the standard type and contained none of this substance. As yet it has not been possible to identify this 'substance *H*', but work in this direction is proceeding.

The 38 samples of *S. sarmentosus* investigated proved more interesting, being divided fairly sharply into two types, one containing sarmentogenin as the only genin present linked to a deoxy sugar, and the other type containing sarverogenin

The accompanying map (Fig. 2) shows the distribution of the two types. It should be noted that the collections in the neighbourhood of Katsina were made by local labour and represent hundreds of individual plants. The relative amounts of sarverogenin and sarmentogenin in the seeds of the 'forest' type vary, but in the typical form are roughly equal; so far we have found no sample of *S. sarmentosus* in which sarmentogenin is completely absent. One or two samples have contained only very small amounts of glycoside, but these

Table 2. *Cardiac aglycones of* Strophanthus *species*

(Where possible the quantities of aglycones contained in the seeds are indicated as a percentage of the weight of the seed. Where no quantitative data are available, the following abbreviations are used. *M*, main constituent; *S*, secondary constituent; *T*, trace only present.)

	Aglycones present						
Species	Strophanthidin (II)	Strophanthidol (III)	Periplogenin (IV)	Sarverogenin (V)	Sarmentogenin (VI)	Ouabagenin (I)	References
Group I							
S. gardeniiflorus	—	—	—	—	—	*M*	—
S. gratus	—	—	—	—	—	3·6*a*	(1)
S. tholloni	—	—	—	—	—	*M*	(2)
Group II							
S. arnoldianus	—	*T*	—	—	—	—	(2)
S. barteri	*M*	—	—	—	—	—	—
S. eminii	0·4*b*	0·18*c*	0·14*d*	—	—	—	(3)
S. gracilis	*S*	*S*	*M*	—	—	—	(4)
S. hispidus	1·6	*S*	*T*	—	—	—	(5)
S. hypoleucus	?	*S*	*M*	—	—	—	(6)
S. kombé	2·4*e*	*S*	*T*	—	—	—	(7)
S. ledienii	*M*	*S*	*S*	—	—	—	(8)
S. mirabilis	0·48	*S*	*S*	—	—	—	(9)
S. mortehanii	?	0·15	?	—	—	—	(2)
S. nicholsonii	0·2	*S*	*S*	—	—	—	(10)
S. preussii	*M*	—	—	—	—	—	—
Group III							
S. amboënsis	—	—	—	*M*	*T*	—	(11)
S. congoensis	—	—	—	1·02	—	—	(2)
S. courmontii	—	—	—	0·22	0·1	—	(12)
S. gerrardi	—	—	—	0·7*f*	0·13*g*	—	(13)
S. intermedius	—	—	—	1·46*h*	—	—	(14, 15)
S. petersianus	—	—	—	—	0·1*g*	—	(16
S. sarmentosus 'forest' form	—	—	—	0·1	0·1	—	(17)
S. sarmentosus 'savannah' form	—	—	—	—	0·26	—	(18)
S. schuchardti	—	—	—	*M*	—	—	(14)
S. welwitschii	—	—	—	0·09*h*	—	—	(19)

Notes

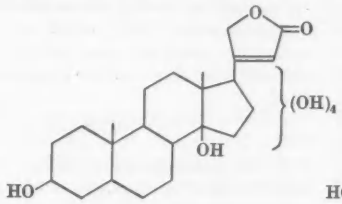
a Content of ouabain.
b Content of cymarin.
c Content of cymarol.
d Content of periplocymarin. A considerable quantity of emicymarin, a difficultly hydrolysable glycoside of periplogenin, is also present.
e Crude, contained some strophanthidol.
f Content of sarveroside. A considerable quantity of other sarverogenin glycosides is also present.
g Content of sarmentocymarin.
h Content of intermedioside. A considerable quantity of other sarverogenin glycoside is also present.

References

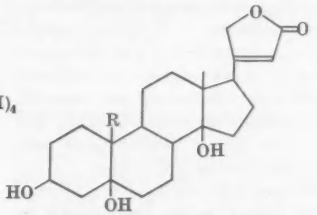
(1) Thoms (1904).
(2) Rothrock, Howe, Florey & Tishler (1950).
(3) Lardon (1950).
(4) Aebi & Reichstein (1951).
(5) Cf. v. Euw & Reichstein (1950*a*).
(6) v. Euw & Reichstein (1950*b*).
(7) Koechlin & Reichstein (1947).
(8) Hess, Speiser, Schindler & Reichstein (1951).
(9) Cf. Bally, Schindler & Reichstein (1952).
(10) Cf. v. Euw & Reichstein (1948).
(11) v. Euw, Hess, Speiser & Reichstein (1951).
(12) Figures for an average specimen of *S. courmontii* investigated by us. Cf. v. Euw & Reichstein (1950*c*).
(13) v. Euw & Reichstein (1950*d*).
(14) v. Euw, Hess *et al.* (1951).
(15) Salmon, Smith & Bywater (1951).
(16) v. Euw & Reichstein (1950*e*).
(17) Cf. Buzas, v. Euw & Reichstein (1950).
(18) Cf. v. Euw, Reber & Reichstein (1951).
(19) v. Euw, Heitz, Hess, Speiser & Reichstein (1952).

were gathered in an extremely unripe condition, so the observation is probably not significant. Two large samples of seed collected in Sierra Leone and the Gambia proved to be similar to the Nigerian 'savannah' type.

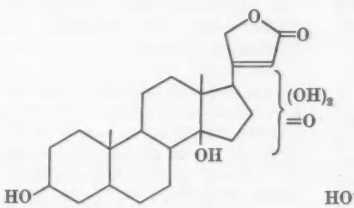
Certain other species of *Strophanthus* have also been investigated by this method, including *S. courmontii* (two specimens), *S. mirabilis*, *S. petersianus*, *S. kombé* (six specimens), *S. amboënsis*, *S. nicholsonii*, *S. barteri*, *S. preussii*, a specimen of *S. intermedius* kindly provided by Prof. Reichstein, and a specimen from Angola which has not been satisfactorily identified, but is probably also *S. intermedius*.



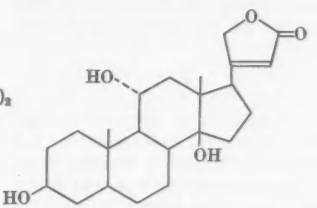
Ouabagenin (I)



R = −CHO Strophanthidin (II)
R = −CH_2OH Strophanthidol (IV)
R = −CH_3 Periplogenin (III)



Sarverogenin (V)



Sarmentogenin (VI)

Of the two specimens of *S. courmontii*, one contained sarverogenin as the only genin attached to a 2-deoxy sugar, whilst the other was very similar to the 'forest' form of *S. sarmentosus* except that it was rather richer in total genin content. The *S. amboënsis* was again similar to the 'forest' form of *S. sarmentosus*, but was considerably richer in total genin, while the *S. petersianus* proved similar to the 'savannah' form of *S. sarmentosus*.

The specimens of *S. kombé* proved to be similar to *S. hispidus*, a trace of periplogenin always being present, together with a fourth substance, 'substance K'. *S. mirabilis* and *S. nicholsonii* were also of the *S. hispidus* type, but here the periplogenin content was greatly increased, being roughly equal to the strophanthidin. *S. barteri* and *S. preussii* proved to be generally similar to the *S. hispidus* type, but contained rather small amounts of glycoside.

The specimen from Angola was particularly interesting, containing about 3% of sarverogenin, no sarmentogenin or only very slight traces, and small amounts of three unidentified substances. The authentic specimen of *S. intermedius*, which occurs very commonly in Angola, gave a chromatogram indistinguishable from that given by the unidentified specimen.

DISCUSSION

From our results and those reported by other workers, especially Prof. T. Reichstein and his colleagues, in numerous papers in the last 12 years, it would appear that the species of the genus *Strophanthus* which have so far been investigated, fall into three well defined groups:

(1) *S. gratus* and the two closely related species, *S. tholloni* and *S. gardeniiflorus*, of which the principal glycoside is ouabain (I). Mr R. D. Meikle of the Royal Botanic Gardens, Kew, tells us that this group has been separated from *Strophanthus* to form the genus *Roupellia*, although this division has apparently not been accepted by all authorities (cf. Baillon, 1891).

(2) *S. arnoldianus*, *S. barteri*, *S. eminii*, *S. gracilis*, *S. hispidus*, *S. hypoleucus*, *S. kombé*, *S. ledienii*, *S. mirabilis*, *S. mortehanii*, *S. nicholsonii* and *S. preussii*. The typical genins of this group are strophanthidin (II) and periplogenin (III), the relative amounts of these two genins varying rather widely from *S. hypoleucus*, which is reported to contain only a little strophanthidin and mainly periplogenin (v. Euw & Reichstein, 1950*b*), to *S. kombé* which contains much strophanthidin and no more than a

minute trace of periplogenin. Strophanthidol (IV) is always present also as a minor constituent. It has been reported that periplogenin does not occur in *S. hispidus* and *S. kombé*, but we have always found traces of it in these plants.

(3) *S. amboënsis*, *S. courmontii*, *S. congoensis*, *S. gerrardi*, *S. intermedius*, *S. petersianus* and its variety *grandiflorus* (syn. *verrucosus*), *S. sarmentosus*, *S. schuchardti* and *S. welwitschii*. In this group the typical genins are sarverogenin (V) and sarmentogenin (VI), although once again the relative proportions of the two substances are variable, *S. intermedius* containing no sarmentogenin, and the 'savannah' form of *S. sarmentosus* and *S. petersianus* containing no sarverogenin.

Although the differences between the two types of *S. sarmentosus* are rather larger than might be expected within a species, Mr Meikle informs us that he can find no botanical justification for a division of the species as at present defined, and it has yet to be proved whether these differences are due to genetic variations or merely to the conditions under which the plants are growing.

Pichon (1949) states that *S. speciosus* and *S. boivinii*, which have been reported by Schindler & Reichstein (1952) as containing gitoxigenin digitaloside and other unidentified substances are not *Strophanthus* species, *S. speciosus* having been placed in the monotypic genus *Christya*, and *S. boivinii*, together with the closely related *S. grevei*, in the genus *Roupellina*.

No *Strophanthus* species has yet been found to contain so much as 1% of sarmentogenin, in contrast to strophanthidin and sarverogenin which both occur in quantities up to 3%.

It is suggested that this extremely simple and rapid method of investigation of *Strophanthus* seeds may well be found suitable as a pharmacognostic test for seed samples to replace the sulphuric acid colour test at present employed (Mathiesen, 1928; Gilg & Schuster, 1919), which has been found to be extremely unreliable, and largely depends on substances other than cardiac glycosides present in the seed.

SUMMARY

1. A paper-chromatographic method for the identification of members of the group of cardiac aglycones has been devised.

2. This method has been applied as a routine analytical method to samples of *Strophanthus* seeds from many species.

3. The results are discussed, and a classification of the genus *Strophanthus*, on purely chemical grounds, is proposed.

We should like to express our thanks to Dr R. K. Callow of this Institute for his interest in this work, to Miss J. Fensome for technical assistance, and to Dr H. M. E. Cardwell for supplying certain of the pure cardiac aglycones; also to Dr D. Fraser, Mr C. C. Kanaar, Mr W. H. Ramsey, Dr G. Taylor, Mr P. Topham, Lt.-Col. Venour, and the Departments of Agriculture of the Gambia and of Sierra Leone, who have aided us by forwarding or collecting certain of the seed samples used in this work.

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The Effect of Salicylate on the Glycosuria and Hyperglycaemia Induced by Cortisone in the Normal Rat

By M. J. H. SMITH
Department of Chemical Pathology, King's College Hospital Medical School, Denmark Hill, London, S.E. 5

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Salicylate has been reported to reduce the glycosuria and blood glucose of alloxan-diabetic rats (Smith, Meade & Bornstein, 1952). Bilateral adrenalectomy also decreases the severity of the diabetes in these animals, and inhibition of the action of adrenal corticoids was considered a possible mechanism of the effect of salicylate. The influence of salicylate on the glycosuria and hyperglycaemia induced by cortisone in normal rats force-fed a high-carbohydrate diet was therefore investigated. The changes in blood glucose and liver glycogen of adrenalectomized rats produced by cortisone, in the presence and absence of salicylate, were also studied.

EXPERIMENTAL

Animals. Male rats of the Wistar strain, maintained on a diet of commercial cubes to a weight of approximately 300 g., were fed a high-carbohydrate diet consisting of: Ostermilk no. 2 (Glaxo), 6 g. (out-of-date hospital stock); Hepovite (Evans Medical Supplies), 1·5 g.; glucose, 9 g.; water to 30 ml.

Increasing amounts of the high-carbohydrate diet were given for a period of 4 days when full feeding was instituted; each rat then received 15 ml. of the diet by stomach tube at 10 a.m. and 6 p.m. Male rats of the same colony, weighing 150–200 g., were used for the experiments on liver-glycogen content.

Analytical methods. Urinary glucose was determined by the method of Benedict (1911) and urinary non-protein nitrogen (N.P.N.) by a micro-Kjeldahl procedure (Harrison, 1949). Liver-glycogen content was measured by the method of Good, Kramer & Somogyi (1933). The glucose in the final stage of the glycogen estimation, and also the blood glucose, was measured by the method of Nelson (1944).

General arrangement of experiments

Effect of salicylate on the glycosuria and hyperglycaemia induced by cortisone in the normal rat. Eight rats maintained on the high-carbohydrate diet were placed in metabolism cages and 24 hr. urine collections made, feeding by stomach tube being performed at 10 a.m. and 6 p.m. daily. After an initial control period each rat received a subcutaneous injection of 2·5 mg. cortisone (cortone acetate, Merck) immediately before force-feeding; after 3 days the dose of cortisone was increased to 5 mg. to ensure an adequate glycosuria. Salicylate was administered as a subcutaneous injection of an arachis-oil suspension of 100 mg./ml. of sodium salicylate. The rats were weighed daily and blood glucose was measured on tail-vein samples in each rat immediately before and at intervals of 2, 4, 6 and 8 hr. after the morning feed on one day of the control period, one day of the period in which cortisone only was given and one day when cortisone and salicylate were being administered.

Effect of cortisone, alone and with salicylate, on the liver-glycogen content of adrenalectomized rats. The rats were bilaterally adrenalectomized and maintained for 4 days after operation on a diet consisting of commercial rat cubes, bread and milk, and were given 0·45 % (w/v) saline to drink. The 48 adrenalectomized rats were divided into seven groups. Three groups, each containing six animals, were killed by stunning at 0, 4 and 6 hr., two groups were injected with 5 mg. of cortisone/rat at 0 hr. and killed at 4 hr. (seven rats) and 6 hr. (11 rats) respectively; in the two remaining groups of six animals each rat received 5 mg. of cortisone and 100 mg. of sodium salicylate at 0 hr., one group was killed at 4 hr. and the other at 6 hr. No preliminary fast was employed, food being removed at 0 hr. The liver-glycogen content of these animals at 0 hr. was within the normal range, whereas a 24 hr. fast reduced it to practically zero (cf. Long, Katzin & Fry, 1940). No preliminary fast was used in the above experiments because the injection of salicylate into rats having a very low liver-glycogen content killed them within 2 hr. Blood-glucose estimations were made immediately before death, and glycogen was estimated in 80–100 mg. portions of liver taken as nearly as possible from anatomically similar lobes immediately after death.

RESULTS

The effect of salicylate on the glycosuria induced by cortisone in the normal rat. The mean 24 hr. glucose outputs of four of the rats are shown in Fig. 1, where it is seen that injection of salicylate markedly reduced the glycosuria which increased again when salicylate was stopped. During the initial control period there was no loss in body weight but a steady loss occurred when cortisone was given. A decrease of approximately 50 g./rat in body weight occurred during this period and was accompanied by an increase in the urinary N.P.N. excretion. In the control period the average daily urinary N.P.N. was 0·26 g. whereas when cortisone was given the rats excreted a daily average of 0·38 g. in the urine. No further decrease in weight occurred when the animals received salicylates but the average urinary N.P.N. (0·37 g.) was not significantly decreased. Four of the

original eight rats died from acute infections during the experiment and are therefore not included in the final results. Similar toxic effects of cortisone in rats, particularly the marked loss in body weight and death from intercurrent infection, have been reported by Ershoff (1951) and Ingle (1952).

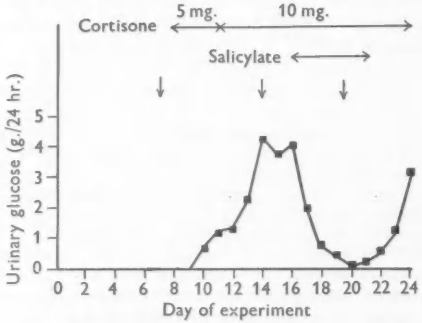

Fig. 1. Mean 24 hr. urinary glucose for a group of four normal rats fed on a high-carbohydrate diet and injected with cortisone and sodium salicylate. Cortisone: 5 or 10 mg. as saline suspension of cortone acetate (Merck) daily; sodium salicylate: 100 mg. in 1 ml. arachis oil daily. The arrows indicate the days on which the blood-sugar curves in Fig. 2 were obtained.

The effect of salicylate on the hyperglycaemia induced in the normal rat by cortisone. Blood glucose was measured in each rat on tail-vein samples immediately before, and 2, 4, 6 and 8 hr. after, the 10 a.m. feed on the 3 days indicated by the arrows in Fig. 1. The results are shown in Fig. 2 where it is seen that the administration of cortisone caused a hyperglycaemic curve whereas the concurrent injection of salicylate markedly reduced the effect.

The effect of cortisone with and without salicylate on the liver-glycogen content and blood glucose of adrenalectomized rats. The liver-glycogen results are shown in Table 1. These results show that fasting caused

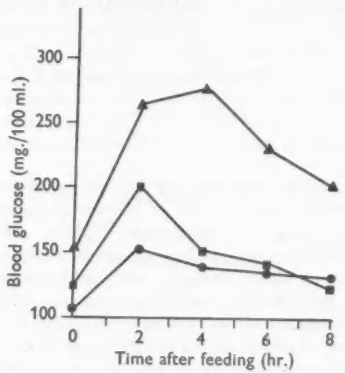

Fig. 2. Mean blood-sugar curves after feeding the rats described in Fig. 1. The animals were fasted for 16 hr. before being given the same amounts of the high-carbohydrate diet by stomach tube. ●, control period, day 7; ▲, cortisone period, day 14; ■, cortisone-salicylate period, day 19.

a significant depletion of glycogen at 6 hr. in the untreated group, whereas cortisone caused a significant deposition of glycogen at 6 hr. The administration of salicylate with the cortisone caused marked depletion of the glycogen at 4 and 6 hr. Comparison of the three groups at 4 hr. showed no significant difference between the untreated and cortisone-treated groups, but the liver glycogen of the salicylate-cortisone-treated group was significantly lower than both. At 6 hr. the liver glycogen of

Table 1. *The effect of cortisone with and without salicylate on the liver glycogen of adrenalectomized rats*
(The liver-glycogen contents are given as g. glycogen/100 g. liver.)

Time killed (hr.)	*A* Untreated: Liver glycogen (%±S.D.)	*A*: No. of rats	*B* Cortisone-treated: Liver glycogen (%±S.D.)	*B*: No. of rats	*C* Cortisone-salicylate-treated: Liver glycogen (%±S.D.)	*C*: No. of rats
0	2·33±0·55	6	—	—	—	—
4	1·77±0·81	6	3·30±2·9	7	0·5±0·12	6
6	1·45±0·70	6	3·99±2·0	11	0·6±0·2	6

Within groups	*A*			*B*			*C*		
	n	*t*	*P*	*n*	*t*	*P*	*n*	*t*	*P*
0 and 4 hr.	10	1·5092	0·2	11	0·8977	0·4	10	7·8829	0·001
0 and 6 hr.	10	3·1196	0·02	15	1·9970	0·1	10	7·2046	0·001

Comparison of liver-glycogen results for groups *A*, *B* and *C*

	A/*B*			*A*/*C*			*B*/*C*		
	n	*t*	*P*	*n*	*t*	*P*	*n*	*t*	*P*
4 hr.	11	1·3881	0·2	10	4·2561	0·01	11	2·6054	0·05
6 hr.	15	3·0494	0·01	10	3·3650	0·01	15	4·1246	0·001

Table 2. *The effect of cortisone with and without salicylate on the fasting blood glucose of adrenalectomized rats*

Time killed (hr.)	A Untreated: Blood glucose (mg./100 ml. ± S.D.)	A: No. of rats	B Cortisone-treated: Blood glucose (mg./100 ml. ± S.D.)	B: No. of rats	C Cortisone-salicylate-treated: Blood glucose (mg./100 ml. ± S.D.)	C: No. of rats
0	125±11	6	—	—	—	—
4	116±9	6	116±2	7	102±26	6
6	106±12	6	123±19	11	95±41	6

Within groups ...	A: *n*	A: *t*	A: *P*	B: *n*	B: *t*	B: *P*	C: *n*	C: *t*	C: *P*
0 and 4 hr.	10	1·6080	0·2	11	2·2733	0·02	10	2·0055	0·1
0 and 6 hr.	10	2·9909	0·01	15	0·2355	0·8	10	1·7254	0·2

Comparison of blood-glucose results for groups *A*, *B* and *C*

	A/B: *n*	A/B: *t*	A/B: *P*	A/C: *n*	A/C: *t*	A/C: *P*	B/C: *n*	B/C: *t*	B/C: *P*
4 hr.	11	0·3069	0·8	10	1·2864	0·3	11	1·6166	0·2
6 hr.	15	1·9972	0·1	10	0·6377	0·6	15	1·9728	0·1

the salicylate-cortisone-treated group was again lower than the other groups and the cortisone-treated group had a significantly raised liver glycogen compared with the untreated group. Cortisone therefore caused deposition of liver glycogen at 6 hr., and the concurrent administration of salicylate not only produced depletion of existing glycogen at 4 and 6 hr., but also prevented the deposition of glycogen by the cortisone at 6 hr.

The blood-glucose results are shown in Table 2. This table shows that fasting reduced the blood glucose at 6 hr. in the untreated group but not in the other two groups. Comparison of the three groups at 4 hr. revealed no significant differences, but at 6 hr. the cortisone-treated group had a significantly higher blood glucose than the other two groups. These results suggest that cortisone prevented the reduction in blood glucose at 6 hr. caused by fasting, but this did not occur when salicylate was given concurrently.

DISCUSSION

The present work shows that in the adrenalectomized rat salicylate prevents the deposition of new glycogen by cortisone and therefore inhibits the increased glycogen synthesis caused by cortisone.

In the normal rat, force-fed a high-carbohydrate diet, salicylate reduced the glycosuria and hyperglycaemia induced by cortisone. The increased urinary-nitrogen excretion when cortisone was given showed that increased gluconeogenesis had occurred, and a possible explanation of the effects of salicylate was an inhibition of this process. This was considered unlikely because the concurrent administration of salicylate almost completely suppressed the glycosuria but caused no significant reduction in the urinary-nitrogen output. In addition Ingle (1941) has shown that in rats maintained on a similar diet and injected with cortisone, only about 1 g. of the daily urinary glucose could be derived from protein. This worker has suggested that cortisone inhibits the utilization of glucose by the tissues, and an interference with this action may provide an explanation of the effects of salicylate on the glycosuria and hyperglycaemia.

Similar considerations apply to the effect of salicylate in reducing the blood glucose and glycosuria of alloxan-diabetic rats (Smith *et al.* 1952) because Stetten, Welt, Ingle & Morley (1951) using [^{14}C] glucose have reported that in these animals the rate of glucose production was only barely increased whereas the rate of oxidation of glucose was markedly reduced.

The antagonistic effects of cortisone and salicylate are also of interest because of the suggestion that the therapeutic effects of salicylates in rheumatic fever are primarily mediated by the pituitary and adrenal glands, leading to the production of adrenal corticosteroids (Hetzel & Hine, 1951). If this hypothesis is correct then salicylates and adrenal corticosteroids, of which cortisone may be considered a typical example, should have the same general effects in the tissues.

SUMMARY

1. The effects of salicylate on the glycosuria and hyperglycaemia induced by cortisone in the normal rat, and of cortisone with and without salicylate on the blood glucose and liver glycogen of the adrenalectomized rat have been studied.

2. Salicylate reduces the glycosuria and hyperglycaemia induced by cortisone in the normal rat. Cortisone causes deposition of liver glycogen in the adrenalectomized rat while the concurrent administration of salicylate produces not only depletion of existing glycogen but also prevents the deposition of new glycogen by the cortisone.

3. The implications of these effects of salicylate are discussed.

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Chemical Studies of Peripheral Nerve During Wallerian Degeneration

4. PHOSPHATASES

By D. M. HOLLINGER, R. J. ROSSITER and H. UPMALIS
Department of Biochemistry, University of Western Ontario, London, Canada

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When a peripheral nerve is cut, that portion of the nerve distal to the point of section soon loses its ability to transmit a nerve impulse. This is associated with a series of changes characteristic of Wallerian degeneration. The axon of each nerve fibre is destroyed, followed by the physical and later by the chemical destruction of the myelin sheath. There is also a cellular proliferation associated with an increase in the concentration of nucleic acid. Accompanying the destruction of the myelin sheath there is a decrease in the concentration of myelin lipids and neurokeratin (see Young, 1942; Abercrombie & Johnson, 1946; Johnson, McNabb & Rossiter, 1949, 1950; Logan, Mannell & Rossiter, 1952, for references).

With the exception of acid phosphomonoesterase, which was studied by Heinzen (1947) and Bodian (1947), chemical determinations of the activity of enzymes in peripheral nerve during Wallerian degeneration have been confined to those enzymes responsible for the destruction or the synthesis of acetylcholine. In this paper, changes in the activity of a number of phosphatases are described. During the degeneration, there is an increase in the rate of hydrolysis of phenylphosphate at pH 4·9 (acid phosphomonoesterase) and of adenosine-5-phosphate at pH 7·5 (5-nucleotidase), a fall in the rate of hydrolysis of phenylphosphate at pH 9·9 (alkaline phosphomonoesterase) and little change in the rate of hydrolysis of adenosinetriphosphate (ATP) at pH 7·2 in the presence of Ca^{++} (adenosinetriphosphatase).

When the nerve is crushed (axonotmesis) rather than sectioned (neurotmesis), a procedure that interrupts the axons and myelin sheaths, but leaves the connective tissue sheaths intact, it degenerates as before. After a short latent period, the degenerating peripheral segment is re-innervated by axon tips from the intact central stump. There is thus a regeneration superimposed upon the degeneration. During the phase of degeneration the changes in the activity of the phosphatases are similar to those following nerve section. During the phase of regeneration the myelin lipids and neurokeratin slowly reappear and the concentration of nucleic acid falls (Burt, McNabb & Rossiter, 1950; Logan *et al.* 1952). There is an increase in the activity of alkaline phosphomonoesterase and adenosinetriphosphatase. While the activity of these two enzymes is increasing, the activity of acid phosphomonoesterases and 5-nucleotidase is falling. A preliminary report of some of these experiments has already appeared (Hollinger & Rossiter, 1952).

METHODS

The sciatic nerve on one side of each of a series of fifty-seven cats was either sectioned or crushed high in the thigh. To minimize the possibility of regeneration in the sectioned nerves, the proximal stump was retracted and sutured to the overlying muscle. In only one instance was there functional or post-mortem evidence of regeneration. This animal was excluded from the series. In the crushed nerves there was a progressive return of function. Microscopic examination of a number of animals killed 96 days after crushing showed that myelination had spread throughout the length of the nerves. In these animals function appeared normal. Details of the operations have already been described (Johnson *et al.* 1949; Burt *et al.* 1950). No attempt was made to control the age, sex or weight of the animals. After intervals of time varying from 1 to 600 days the animals were killed and the distal degenerating or regenerating segment of nerve was removed. At the same time a similar length of sciatic nerve from the opposite side was removed to serve as a control. Each nerve was cleaned of adherent fatty and epineural connective tissue, rapidly weighed, frozen in liquid nitrogen and ground to a fine powder in a mortar. Homogenates of the required dilution (1:20) were prepared in ice-cold isotonic sodium chloride using an ice-cooled Potter-Elvehjem type glass homogenizer. Because of its high collagen content, the sciatic nerve of the cat is a difficult tissue to homogenize, but good homogenates may be obtained from the frozen powder. Experiments with brain tissue, which can readily be homogenized directly, showed that the freezing had no effect on the enzymes under investigation.

Enzyme determinations

For each determination, 0·2 ml. of the homogenate was taken, except for the determination of adenosinetriphosphatase activity, where 0·1 ml. sufficed. All determinations and the appropriate blanks were run in duplicate.

Phosphomonoesterase activity. This was determined by a modification of the original method of King & Armstrong (1934). Alkaline phosphomonoesterase was determined in carbonate-bicarbonate buffer pH 9·9, as described by King, Haslewood, Delory & Beall (1942) and acid phosphomonoesterase in citrate buffer pH 4·9, as described by Watkinson, Delory, King & Haddow (1944). The reaction mixture contained $MgCl_2$ in a final concentration of 0·001 M, which is optimal for many phosphatases (Jenner & Kay, 1931). The reaction mixture was incubated for 1 hr. at 37°. The quantity of free phenol liberated from the substrate phenylphosphate (0·004 M) was determined by the method of Folin & Ciocalteu (1927). The results are reported in terms of μg. P liberated/hr./mg. wet weight of nerve, based on the assumption that inorganic phosphate and free phenol were liberated in equimolecular proportions.

5-Nucleotidase activity. This was determined by the method of Reis (1937, 1951). The reaction mixture, buffered with 0·05 M-diethylbarbiturate buffer, pH 7·5, was incubated for 30 min. at 37°. Following Reis (1951), the results are reported in terms of μg. P liberated from 0·00083 M-adenosine-5-monophosphate (Schwartz)/hr./mg. wet weight of nerve.

Adenosinetriphosphatase activity. This was determined by the method of DuBois & Potter (1943). The reaction mixture, which contained 0·01 M-$CaCl_2$ and was buffered with 0·05 M-diethylbarbiturate buffer, pH 7·2, was incubated for 15 min. at 37°. The results are reported in terms of μg. P liberated from 0·003 M-ATP (Schwartz)/hr./mg. wet weight of nerve.

Recording of results

Because of the great increase in the wet weight of degenerating and regenerating nerves, we have, as in previous publications (Johnson *et al.* 1949; Burt *et al.* 1950; Logan *et al.* 1952), expressed the results in terms of the wet weight of a similar length of the control nerve of the opposite side. This is equivalent to expressing the results in terms of the fresh weight of the nerve before it had been sectioned or crushed, i.e. at zero time. To assess the validity of this procedure, the activity of each of the enzymes was determined in both the right and the left sciatic nerve of six cats that were not subjected to any operative procedure. In no instance did the enzyme activity in the right nerve differ significantly from that in the left, the standard error of the mean percentage difference being $\pm 9{\cdot}1\%$ for the acid phosphomonoesterase, $\pm 6{\cdot}1\%$ for the alkaline phosphomonoesterase, $\pm 9{\cdot}0\%$ for the 5-nucleotidase and $\pm 5{\cdot}7\%$ for the adenosinetriphosphatase.

RESULTS

Acid phosphomonoesterase. Table 1 shows that 16 days after either nerve section or nerve crush there was an increase in the acid phosphomonoesterase activity. After each type of operation the mean increase was of the order of 300%. Fig. 1 shows the time course of the mean change in acid phosphomonoesterase activity for the first 96 days after nerve section. The enzyme activity was greatest 16 days after the operation. As early as 4 days, the mean activity in the degenerating nerve was significantly ($P < 0{\cdot}01$) greater than that in the control nerves, but 24 and 48 hr. after the operation the difference between the mean activities of the two sides was not statistically significant.

When the nerve was crushed there was also an increase in the mean acid phosphomonoesterase activity (Fig. 2). During the first 96 days the figures were not significantly different from those obtained after nerve section. After 175 days the mean activity in the crushed nerve was still significantly ($P < 0{\cdot}01$) greater than that of the control nerves. Three animals were kept for 600 days after the operation. In each of them the activity in the crushed nerve was greater than that in the control nerve of the opposite side, but the percentage increase varied so greatly from animal to animal (11–71%) that the mean increase of $37\% \pm 18$ was only of doubtful significance ($P = 0{\cdot}1$).

Alkaline phosphomonoesterase. Whereas the activity of acid phosphomonoesterase increased after either nerve section or nerve crush, the activity of alkaline phosphomonoesterase decreased. Table 2 shows that by 16 days, after either type of operation, the mean enzyme activity in the degenerating nerves had fallen to less than 50% of the

Table 1. *Acid phosphomonoesterase activity of the sciatic nerve of the cat 16 days after either nerve section or nerve crush*

(Substrate, phenylphosphate. pH 4·9. Incubation time, 1 hr. Temp. 37°.)

Enzyme activity (μg. P/hr./mg. wet wt.)			
Normal	After nerve section (16 days)	Increase	Percentage increase
0·57	2·40	1·83	320
0·44	1·39	0·95	217
0·64	1·69	1·05	164
0·32	1·49	1·17	366
0·27	0·97	0·70	260
0·27	1·99	1·72	637
0·41	1·20	0·79	192

Mean (±S.E.M.) = **308** (±61)

Enzyme activity (μg. P/hr./mg. wet wt.)			
Normal	After nerve crush (16 days)	Increase	Percentage increase
0·28	0·98	0·70	249
0·30	1·46	1·16	387
0·37	1·24	0·87	234

Mean (±S.E.M.) = **290** (±49)

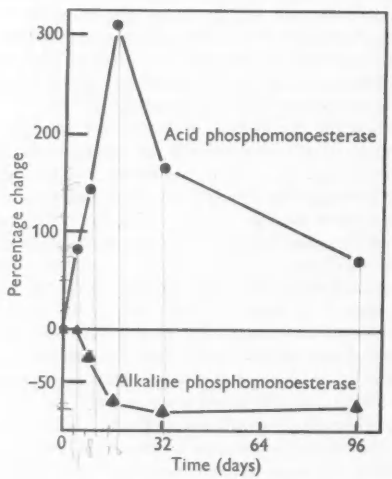

Fig. 1. Mean percentage change in the activity of acid phosphomonoesterase (●–●–●) and alkaline phosphomonoesterase (▲–▲–▲) in the sciatic nerve of the cat after nerve section. Each point is the mean of three or more determinations.

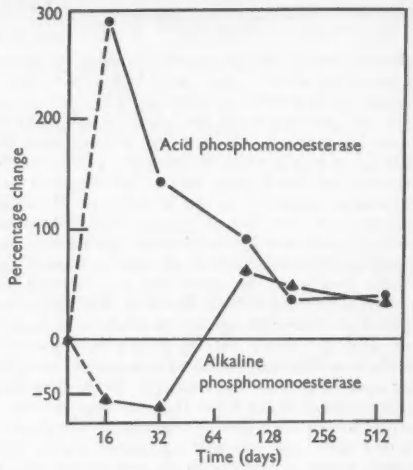

Fig. 2. Mean percentage change in the activity of acid phosphomonoesterase (●–●–●) and alkaline phosphomonoesterase (▲–▲–▲) in the sciatic nerve of the cat after nerve crush. Each point is the mean of three or more determinations.

Table 2. *Alkaline phosphomonoesterase activity of the sciatic nerve of the cat 16 days after either nerve section or nerve crush*

(Substrate, phenylphosphate. pH 9·9. Incubation time, 1 hr. Temp. 37°.)

Enzyme activity (μg. P/hr./mg. wet wt.)			
Normal	After nerve section (16 days)	Decrease	Percentage decrease
0·30	0·10	0·20	68
0·49	0·00	0·49	100
0·51	0·11	0·40	79
0·55	0·26	0·29	52
0·25	0·05	0·20	79
0·40	0·14	0·26	65
0·33	0·12	0·21	63

Mean (±S.E.M.) = **72** (±6)

Enzyme activity (μg. P/hr./mg. wet wt.)			
Normal	After nerve crush (16 days)	Decrease	Percentage decrease
0·15	0·07	0·08	55
1·06	0·63	0·43	41
0·49	0·08	0·41	83
0·73	0·53	0·20	27
0·23	0·12	0·11	49
0·43	0·09	0·34	79

Mean (±S.E.M.) = **56** (±9)

figure for the control nerves. Fig. 1 shows that after section the mean alkaline phosphomonoesterase activity fell rapidly during the period 4–16 days and was still at this low level after 96 days.

Fig. 2 shows the mean changes in the alkaline phosphomonoesterase activity when the nerve was crushed. The enzyme activity decreased during the first 32 days, as it did after nerve section. It then rose sharply, so that by 96 days the mean activity in the crushed nerves was some 60% greater than that in the controls. After 175 days the activity of the alkaline phosphomonoesterase in the crushed nerve was still high ($P<0.01$). In each of the three animals kept for 600 days the activity in the crushed nerves was greater than that of the control nerves but, as was found for the acid phosphomonoesterase, the percentage increase varied so much from animal to animal (12–59%) that the mean increase of 30% ± 15 was only of doubtful significance ($P=0.1$).

Table 3. *5-Nucleotidase activity of the sciatic nerve of the cat 16 days after either nerve section or nerve crush*

(Substrate, adenosine-5-phosphate. pH 7·5. Incubation time, 30 min. Temp. 37°.)

Enzyme activity (μg. P/hr./mg. wet wt.)			
Normal	After nerve section (16 days)	Increase	Percentage increase
2·23	3·47	1·24	56
2·77	3·15	0·38	14
3·12	5·90	2·78	89
2·87	3·98	1·11	39
2·15	3·22	1·07	50
3·43	5·44	2·01	59
1·38	1·71	0·33	24
		Mean (±S.E.M.)=	47 (±9)

Enzyme activity (μg. P/hr./mg. wet wt.)			
Normal	After nerve crush (16 days)	Increase	Percentage increase
1·19	1·91	0·72	61
1·46	2·71	1·25	86
1·71	3·03	1·32	77
3·40	3·23	−0·17	−5
4·15	4·27	0·12	3
2·54	4·73	2·19	86
		Mean (±S.E.M.)=	51 (±17)

5-Nucleotidase. Table 3 shows that in normal nerves inorganic phosphate was liberated much more rapidly from adenosine-5-phosphate at pH 7·5 than from phenylphosphate at pH 4·9 or 9·9. The mean activity of the 5-nucleotidase also increased 16 days after either nerve section or nerve crush. The activity of this enzyme in the intact nerve was found to be more variable than that of the non-specific phosphomonoesterases. The increase after the operation was also more irregular.

The time course of the mean change in 5-nucleotidase activity after either type of operation is shown in Fig. 3. In contrast to the findings for the acid phosphomonoesterase, the activity of 5-nucleotidase was greater at 32 days than at 16 days. Because of the great animal to animal variation with this enzyme, the difference between the mean activities of the crushed and sectioned nerves at 96 days is of doubtful significance ($P=0.1$).

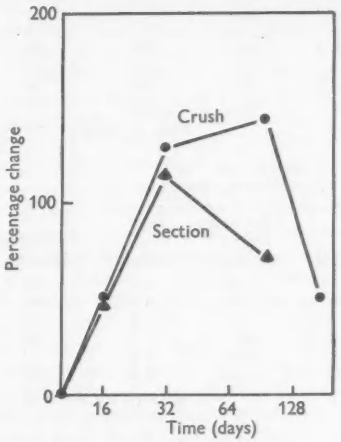

Fig. 3. Mean percentage change in the activity of 5-nucleotidase in the sciatic nerve of the cat after either nerve section (▲–▲–▲) or nerve crush (●–●–●). Each point is the mean of three or more determinations.

Adenosinetriphosphatase. In normal nerves inorganic phosphate was liberated from ATP more rapidly than from adenosine-5-phosphate (Table 4). There was little change in this activity 16 days after nerve section. Again there was considerable variation from animal to animal for both the intact nerves and the sectioned nerves. On the other hand, when the nerve was crushed there was a consistent increase in activity ($P<0.01$) 16 days after the operation.

Fig. 4 shows the time course of the change in the rate at which inorganic phosphate was liberated from ATP. With the sectioned nerves the mean increase was at no time statistically significant. After nerve crush the mean activity at 96 days was greater than that at 16 days. In this respect adenosinetriphosphatase resembled alkaline phosphomonoesterase (cf. Fig. 2). By 175 days the increase was less, but the mean activity in the crushed nerves was still significantly ($P<0.01$) greater than that in the controls.

Table 4. *Adenosinetriphosphatase activity of the sciatic nerve of the cat 16 days after either nerve section or nerve crush*

(Substrate, adenosinetriphosphate (0·003 M). pH 7·2. Incubation time 15 min. Temp. 37°.)

Enzyme activity (μg. P/hr./mg. wet wt.)			
Normal	After nerve section (16 days)	Increase	Percentage increase
13·1	12·8	−0·3	−2
14·3	14·4	0·1	1
14·8	20·6	5·8	39
15·8	22·1	6·3	40
17·2	15·3	−1·9	−11
14·5	20·1	5·6	39
12·4	14·4	2·0	16
		Mean (±S.E.M.)=	17 (±9)

Enzyme activity (μg. P/hr./mg. wet wt.)			
Normal	After nerve crush (16 days)	Increase	Percentage increase
2·6	5·3	2·7	104
9·6	14·4	4·8	50
6·6	12·1	5·5	83
13·6	20·8	7·2	53
13·6	20·0	6·4	47
16·8	25·6	8·8	52
		Mean (±S.E.M.)=	65 (±10)

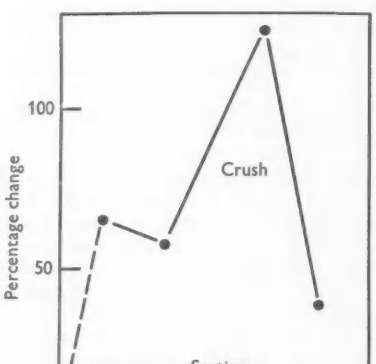

Fig. 4. Mean percentage change in the activity of adenosinetriphosphatase in the sciatic nerve of the cat after either nerve section (▲–▲–▲) or nerve crush (●–●–●). Each point is the mean of three or more determinations.

DISCUSSION

Acid phosphomonoesterase. The mean acid phosphomonoesterase activity in cat sciatic nerve was only one-third of the value found by McNabb (1951) for the cerebral cortex of the dog, although it was of the same order as that reported by Reis (1951) for human cerebral cortex.

The observed increase in acid phosphomonoesterase activity during Wallerian degeneration confirms the previous findings of Bodian (1947) and Heinzen (1947), but the biphasic curve described by the latter worker was not evident. To relate, as

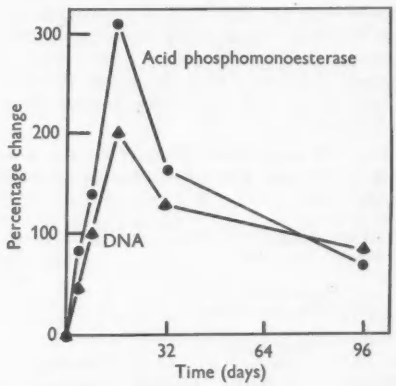

Fig. 5. Mean percentage change in the activity of acid phosphomonoesterase (●–●–●) and in the concentration of deoxypentosenucleic acid (DNA) (▲–▲–▲) in the sciatic nerve of the cat after nerve section. Each point is the mean of three or more determinations.

Heinzen (1947) has done, the initial increase in acid phosphomonoesterase activity to the chemical destruction of myelin would not appear to be justified, for in cat nerve the chemical, as opposed to the physical, destruction of myelin does not occur until after the first week of degeneration (Johnson *et al.* 1949, 1950). It would be reasonable to associate the increase in acid phosphomonoesterase activity with the increase in the number of Schwann cells and endoneurial cells (chiefly fibrocytes and macrophages) found by Abercrombie & Johnson (1946) in the degenerating sciatic nerve of the rabbit, for the increase takes place at a similar time and is of the same order of magnitude. With the cellular proliferation there is also an increase in both pentosenucleic acid and deoxypentosenucleic acid (Logan *et al.* 1952). Fig. 5 shows that after nerve section the changes in the activity of acid phosphomonoesterase follow the same general pattern as the changes in the concentration of deoxypentosenucleic acid. If the mean quantity of deoxypentosenucleic acid per

diploid cell is constant for any one species (see Davidson, Leslie & White, 1951, for references), the activity of acid phosphomonoesterase per cell does not change greatly during the degeneration. The curves representing the changes with time of the concentration of deoxypentosenucleic acid and of the activity of acid phosphomonoesterase (Fig. 5) are of the same general form as that reported by Abercrombie & Johnson (1946) for the total number of cell nuclei in the degenerating sciatic nerve of the rabbit.

Using histochemical methods, Wolf, Kabat & Newman (1943) detected the presence of an acid phosphatase in the axons of normal peripheral nerve, slight activity in the nuclei of the Schwann cells and endoneurial cells, but none in their cytoplasm and none in the myelin sheath. These results were confirmed by Smith (1948). Heinzen (1947) and Bodian (1947), although they found large increases in the acid phosphomonoesterase activity of degenerating nerve determined chemically, could find no comparable change in histological sections examined by the methods of Gomori (1939). In addition, both Lassek & Beuker (1947) and Smith (1948) reported the disappearance of the strong acid phosphatase reaction of the axon during Wallerian degeneration. This disturbing difference between the results obtained by histochemical and chemical methods may in part be technical, for Bartelmez & Bensley (1947) reported results more in keeping with the chemical findings when fresh, rather than fixed, preparations were used.

Alkaline phosphomonoesterase. We are aware of no previous reports on the chemical determination of alkaline phosphomonoesterase activity in normal or in degenerating and regenerating peripheral nerves. The mean alkaline phosphomonoesterase activity of cat sciatic nerve was of the same order as that found for acid phosphomonoesterase. It was considerably less than that reported by McNabb (1951) for the cerebral cortex of the dog, but it was of the same order as that found by Reis (1951) for the cerebral cortex of man. The fall in alkaline phosphomonoesterase activity during Wallerian degeneration was quite rapid and may be compared to the fall in the activity of the true or specific cholinesterase that occurs in peripheral nerve after nerve section (Sawyer, 1946) and in the superior cervical ganglion after denervation (Sawyer & Hollinshead, 1945; Hard, Peterson & Fox, 1951). Similar changes occur in the enzyme system responsible for the synthesis of acetylcholine (Feldberg, 1943; Nachmansohn, John & Berman, 1946; Banister & Scrase, 1950). It is possible that alkaline phosphomonoesterase, like true cholinesterase, is associated with the axon of a nerve fibre rather than with the sheath tissues.

When the nerve was crushed, rather than sectioned, there was a remarkable increase in alkaline phosphomonoesterase activity during the regeneration phase (Table 2). At 96 days, when the alkaline phosphatase activity was at its height, functional activity appeared to have been restored. Despite this, it is well known that at this time regeneration is far from complete (Young, 1942) and neither the myelin lipids (Johnson *et al.* 1949) nor the nucleic acids (Logan *et al.* 1952) have returned to normal. It is possible that the increase in alkaline phosphomonoesterase activity is associated with the regeneration process and with remyelination.

Although the intact sciatic nerve of the cat undoubtedly contains alkaline phosphomonoesterase and the activity of this enzyme decreases greatly during Wallerian degeneration, this activity is difficult to detect with the standard histochemical tests. Gomori (1941) stated the belief that the only histochemically detectable site of alkaline phosphatase activity in the nervous system was in the blood vessels, an observation confirmed by Landow, Kabat & Newman (1942) for the sciatic nerve of the cat. That the fall in alkaline phosphomonoesterase activity in degenerating nerve is due to the disappearance of the blood vessels would appear to be unlikely, since Abercrombie & Johnson (1946) reported that the number of blood-vessel nuclei (endothelial and smooth muscle) in the degenerating sciatic nerve of the rabbit actually increases during degeneration, although the increase is small compared with the increase in the number of tubal and endoneurial cells. More recently Newman, Feigin, Wolf & Kabat (1950) reported a slight alkaline phosphatase reaction in the axon of nerve fibres from several species; Marchant (1949) described a slight activity, sporadically distributed in 'streaks', in intact rabbit nerves, and a strong reaction in the nuclei of most cell types and also in the cytoplasm of the Schwann cells and in the myelin debris of nerves undergoing Wallerian degeneration.

It is surprising that histochemical studies indicate that there is a decrease in the activity of acid phosphatase and an increase in the activity of alkaline phosphatase in peripheral nerve during Wallerian degeneration, whereas chemical studies indicate the direct opposite. These differences between the results obtained by chemical and histochemical techniques merit further study.

5-Nucleotidase. Reis (1937) reported the presence of a 5-nucleotidase in brain and peripheral nerve, specific for substrates such as adenosine-5-phosphate and inosine-5-phosphate. Thus the presence of 5-nucleotidase in nerve homogenates will not interfere with the determination of the activity of the non-specific acid or alkaline phosphomonoesterases, but since adenosine-5-phosphate is hydrolysed by acid and alkaline phosphomonoesterase these enzymes, if present in the homogenates in appreciable

concentration, will interfere with the determination of the 5-nucleotidase activity. Tables 1, 2 and 4 show that with normal and degenerating nerve the rate at which inorganic phosphate is produced from phenylphosphate, even at pH 4·9 or 9·9, is considerably less than the rate at which it is produced from adenosine-5-phosphate at pH 7·5. Since the 5-nucleotidase activity was determined at pH 7·5, a pH far from the optimum for either acid or alkaline phosphomonoesterase, the interference due to non-specific phosphomonoesterase activity would be minimal (Reis, 1951). That during nerve degeneration there is a true increase in the activity of a specific 5-nucleotidase is shown by the observation that inorganic phosphate was formed at a greater rate 32 days after the operation than at 16 days (Fig. 3), while at 32 days the rate of hydrolysis of phenylphosphate at pH 4·9 or 9·9 was less than that at 16 days (Fig. 1).

5-Nucleotidase has been demonstrated histochemically in the interstitial substance of the central nervous system and in the axons of peripheral nerve by Newman *et al.* (1950). The activity found in the undegenerated sciatic nerve of the cat is a little greater, and that in the degenerating nerve considerably greater, than the activity reported by Reis (1951) for human brain.

Adenosinetriphosphatase. Adenosinetriphosphatase activity was reported in squid nerve by Libet (1948) and in frog nerve by Sharples, Grundfest & Nachmansohn (1948). The finding that, in homogenates of the sciatic nerve of the cat, inorganic phosphate is liberated from ATP much more rapidly than from phenylphosphate or adenosine-5-phosphate suggests that this tissue also contains an enzyme specific for ATP. The adenosinetriphosphatase activity was determined in the presence of added Ca^{++} but with no added Mg^{++}. Adenosinetriphosphatases vary greatly in their sensitivity to added Mg^{++} and Ca^{++}. It should be noted that Meyerhof & Wilson (1947) found that the addition of Mg^{++} increased the adenosinetriphosphatase activity of brain homogenates, and that a Mg^{++}-sensitive enzyme, capable of liberating inorganic phosphate from ATP, was recently prepared from pig brain by Binkley & Olson (1950). The activity found for the intact cat nerve is only about one-half of that found for rat brain by DuBois & Potter (1943).

Since adenosinetriphosphatases do not liberate inorganic phosphate from phenylphosphate or adenosine-5-phosphate, it is unlikely that the presence of adenosinetriphosphatase in the nerve homogenates will interfere with the determination of the other phosphatases. A more important problem is the possibility that the non-specific phosphomonoesterases and 5-nucleotidase may interfere with the determination of the adenosinetriphosphatase. Although there is evidence that acid phosphomonoesterase is inactive towards ATP (McLeod & Summerson, 1946; Meister, 1948) purified alkaline phosphomonoesterase has been shown to be active (Liebknecht, 1939; Schmidt & Thannhauser, 1943). Under the conditions of the adenosinetriphosphatase test, the non-specific phosphomonoesterases are at a pH far from optimal, but the production of inorganic phosphate from ATP may be in excess of that caused by the activity of adenosinetriphosphatase alone. This discrepancy may be greater in degenerating and regenerating nerves, where the activity of the non-specific phosphomonoesterases and 5-nucleotidase is increased.

SUMMARY

1. The rate of hydrolysis of phenylphosphate at pH 4·9 (acid phosphomonoesterase), phenylphosphate at pH 9·9 (alkaline phosphomonoesterase), adenosine-5-phosphate at pH 7·5 (5-nucleotidase) and adenosinetriphosphate at pH 7·2 in the presence of Ca^{++} (adenosinetriphosphatase) was determined in the sciatic nerve of the cat at intervals of time from 2 to 96 days after nerve section and from 16 to 600 days after nerve crush.

2. After either nerve section or nerve crush the activity of the acid phosphomonoesterase increased rapidly, reaching a maximum at 16 days after the operation and then decreased slowly. The increase was of the same order and occurred at the same time as the increase in the concentration of deoxypentosenucleic acid previously reported.

3. After nerve section the activity of the alkaline phosphomonoesterase decreased rapidly during the period 4–16 days after the operation and then remained at a low level until 96 days. After nerve crush the activity decreased during the first 32 days and then increased, so that by 96 and 175 days the activity in the crushed nerves was greater than that in the controls.

4. The activity of the 5-nucleotidase increased after either type of operation, reaching a maximum in 32 days after nerve section and 96 days after nerve crush.

5. The rate of hydrolysis of adenosinetriphosphate did not change after nerve section, but increased after nerve crush, reaching a maximum 96 days after the operation.

6. These results have been discussed in relation to the morphological and histochemical changes known to occur in degenerating and regenerating nerves.

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Chemical Studies of Peripheral Nerve During Wallerian Degeneration

5. β-GLUCURONIDASE

By D. M. HOLLINGER and R. J. ROSSITER

Department of Biochemistry, University of Western Ontario, London, Canada

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In an endeavour to outline more fully the chemical changes that take place in a peripheral nerve after nerve section or nerve crush, measurements have been made of the activity of the enzyme β-glucuronidase.

Previous papers in this series have described the changes that occur in the concentration of lipids (Johnson, McNabb & Rossiter, 1949; Burt, McNabb & Rossiter, 1950) and protein-bound phosphorus compounds (Logan, Mannell & Rossiter, 1952) and in the activity of phosphatases (Hollinger, Rossiter & Upmalis, 1952) in peripheral nerve after either nerve section or nerve crush.

METHODS

The sciatic nerve on one side of each of a series of fifty-six cats was either sectioned or crushed high in the thigh. Details of the operations have already been described (Johnson *et al.* 1949; Burt *et al.* 1950). No attempt was made to control the age, sex or weight of the animals. After intervals of time varying from 1 to 600 days the animals were killed and the distal degenerating or regenerating segment of the nerve was removed. At the same time a similar length of nerve of the opposite side was taken to serve as a control. The two pieces of nerve were homogenized in 10 ml. isotonic saline as described by Hollinger *et al.* (1952).

Determination of β-glucuronidase activity. The β-glucuronidase activity of portions of the homogenate was determined by the method of Fishman, Springer & Brunetti (1948) using the biosynthetic substrate, phenolphthalein mono-β-glucuronide, prepared as described by these workers. The reaction mixture contained 0·1 ml. homogenate (diluted by a factor of 3–5 for nerves of high activity), 1 ml. 0·1 M-acetate buffer, pH 4·5, and 0·1 ml. 0·01 M-phenolphthalein mono-β-glucuronide. This mixture was incubated at 37° for 4 hr., at the end of which time the reaction was stopped by the addition of 1 ml. 20% (w/v) trichloroacetic acid. A control tube containing enzyme preparation and buffer, but no substrate, was incubated for the same length of time. Substrate was added to this tube immediately before the addition of the trichloroacetic acid. The determination was completed as described by Fishman *et al.* (1948), the test and control being read against a blank containing buffer and trichloroacetic acid in a Coleman universal spectrophotometer at 540 mμ. A standard containing 20 μg. phenolphthalein/tube was read with each set of determinations. The test was always run in duplicate.

It will be noted that the determination was done on a portion of the whole homogenate and not, as is sometimes customary (Kerr & Levvy, 1951; Walker & Levvy, 1951), on a tissue extract. Under our conditions the rate of production of free phenolphthalein was constant throughout the 4 hr. incubation period. Each tube contained no more than 5 mg. wet tissue in a total volume of 1·2 ml. and for some of the degenerating nerves this was reduced to as little as 1 mg. In this range, the enzyme activity was not a function of the concentration of the homogenate, nor was it increased by the addition of detergents.

Recording of results. The results are reported in terms of phenolphthalein (in μg.) liberated/hr./mg. wet wt. of nerve. These units are equivalent to the glucuronidase units of Fishman *et al.* (1948). Because of the great increase in the wet weight of the degenerating and regenerating nerves, we have, as in the paper by Hollinger *et al.* (1952), expressed the results in terms of the wet weight of a similar length of the control nerve of the opposite side. This is equivalent to expressing the results in terms of the fresh weight of the nerve before it had been sectioned or crushed, i.e. at zero time. In six animals not subjected to any operative procedure the activity of the right nerve did not differ significantly from that of the left when expressed on this basis.

RESULTS

Table 1 shows that 16 days after the nerve was either sectioned or crushed the mean activity of the β-glucuronidase had increased by a factor of 30–40. The mean increase for the crushed nerves was not significantly different from that for the sectioned nerves. Fig. 1 shows the time course of the mean percentage change in the activity of the enzyme after nerve section. On the same figure is shown the much smaller increase in the activity of acid phosphomonoesterase previously reported by Hollinger *et al.* (1952). The similar changes after nerve crush are shown in Fig. 2. At no time up to 96 days did the values for the crushed nerves significantly differ from those for the sectioned nerves. At both 170–180 and 600 days the β-glucuronidase activity of the crushed nerves was still significantly greater than that of the control nerves. At 600 days the mean increase was 30% ± 4.

Table 1. *β-Glucuronidase activity of the sciatic nerve of the cat 16 days after either nerve section or nerve crush*

(Substrate, phenolphthalein mono-β-glucuronide. pH 4·5. Incubation time, 4 hr. Temp. 37°.)

Enzyme activity (μg. phenolphthalein/hr./mg. wet wt.)			
Normal	After nerve section (16 days)	Increase	Percentage increase
0·45	9·45	9·00	2000
0·43	11·15	10·72	2470
0·41	19·45	19·04	4650
0·37	10·00	9·63	2610
0·35	15·30	14·95	4270
0·26	11·60	11·34	4350
		Mean (±S.E.M.) = **3400** ± 480	

Enzyme activity (μg. phenolphthalein/hr./mg. wet wt.)			
Normal	After nerve crush (16 days)	Increase	Percentage increase
0·31	7·10	6·79	2180
0·25	13·75	13·50	5400
0·30	11·95	11·65	3880
		Mean (±S.E.M.) = **3820** ± 890	

Logan *et al.* (1952) described an increase in the concentration of both deoxypentosenucleic acid and pentosenucleic acid after nerve section. In Table 2 the mean activity of β-glucuronidase in normal nerves and that in degenerating nerves 16 days after nerve section are compared with the activities of acid phosphomonoesterase (Hollinger *et al.* 1952) and the concentrations of deoxypentosenucleic acid (Logan *et al.* 1952) at corresponding time intervals. It can be seen that the increase in the activity of acid phosphomonoesterase was of the same order as the increase in the concentration of deoxypentosenucleic acid, but that the increase in the activity of β-glucuronidase was considerably greater.

If the mean quantity of deoxypentosenucleic acid per diploid cell is of the same order for any given species (evidence summarized by Davidson, Leslie & White, 1951) the amount of deoxypentosenucleic acid gives an estimate of the total number of cells throughout the entire length of the nerve. Table 2 shows that, whereas the mean acid phosphomonoesterase activity per μg. deoxypentosenucleic acid P had increased only slightly (48%) at the end of 16 days, the mean β-glucuronidase activity per μg. deoxypentosenucleic acid P, i.e. the mean activity per cell, had increased by over 1100%.

DISCUSSION

As far as we are aware, this is the first report on the β-glucuronidase activity of peripheral nerve. The value for cat sciatic nerve (0·35 μg. phenolphthalein/hr./mg. wet weight) corresponds to 0·35 Fishman units/mg. This figure is less than that reported by Talalay, Fishman & Huggins (1946) for most tissues, but the figure of 12·8 Fishman units/mg. for degenerating nerve is of the same order as that found for rat liver by these workers and is greater than the figures they report for all other rat tissues with the exception of spleen. The activity in the sciatic nerve of the cat is eight times greater than that reported by McNabb (1951) for the brain of the dog.

The great increase in the activity of β-glucuronidase during Wallerian degeneration is of interest, since it has been suggested that β-glucuronidase activity may be associated with cellular proliferation generally (Levvy, Kerr & Campbell, 1948; Kerr, Campbell & Levvy, 1949, 1950). It is also noteworthy that Fishman (1947) showed that the administration of oestrogen to castrated female mice caused an increase in the activity of β-glucuronidase in the uterus. Fishman and his colleagues have also demonstrated a high β-glucuronidase activity in

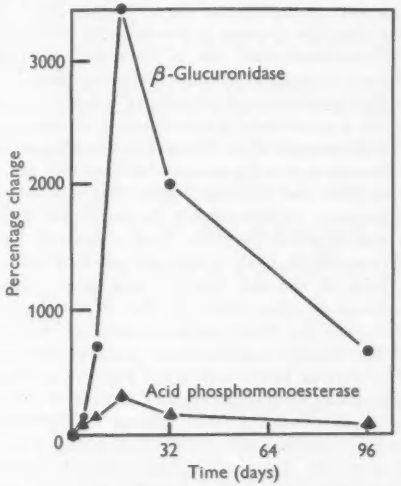

Fig. 1. Mean percentage change in the activity of β-glucuronidase (●–●–●) and acid phosphomonoesterase (▲–▲–▲) in the sciatic nerve of the cat after nerve section. Each point is the mean of three or more determinations.

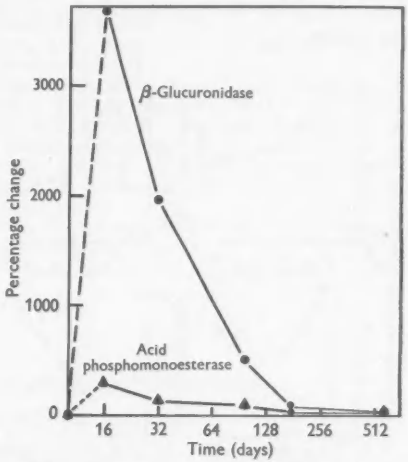

Fig. 2. Mean percentage change in the activity of β-glucuronidase (●–●–●) and acid phosphomonoesterase (▲–▲–▲) in the sciatic nerve of the cat after nerve crush. Each point is the mean of three or more determinations.

Table 2. *β-Glucuronidase activity, acid phosphomonoesterase activity and deoxypentosenucleic acid concentration in the sciatic nerve of the cat* 16 *days after nerve section*

(Mean ± S.E.M. Figures in parentheses indicate the number of animals in each group.)

	Normal	After nerve section (16 days)	Percentage increase
β-Glucuronidase (μg. phenolphthalein/hr./mg. wet wt.)	0·35 ± 0·013 (56)	12·8 ± 1·60 (6)	3560
Acid phosphomonoesterase (μg. P/hr./mg. wet wt.)	0·37 ± 0·013 (56)	1·64 ± 0·188 (7)	344
Deoxypentosenucleic acid (μg. P/100 mg. wet wt.)	4·8 ± 0·1 (52)	14·4 ± 0·9 (6)	200
β-Glucuronidase (μg. phenolphthalein/hr./μg. deoxypentosenucleic acid P)	7·3	88·9	1120
Acid phosphomonoesterase (μg. P/hr./μg. deoxypentosenucleic acid P)	7·7	11·4	48

certain tumours (Fishman & Anlyan, 1947; Fishman Anlyan & Gordon, 1947). These results were later confirmed and extended by Odell & Burt (1949). In addition, Bernard & Odell (1950) reported changes in the uterus and ovary of the rat during pregnancy and Odell & Fishman (1950) described cyclic changes in the β-glucuronidase activity of specimens of endometrium obtained at biopsy during the different phases of the menstrual cycle in the human female.

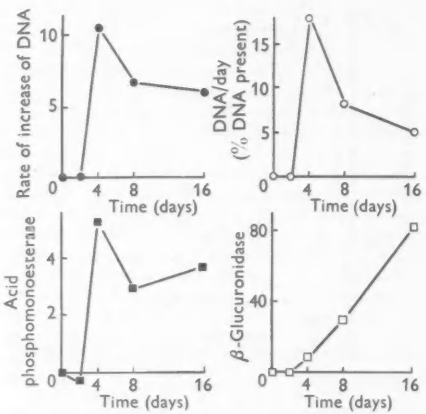

Fig. 3. Comparison of rate of increase of deoxypentosenucleic acid with increase of enzyme activity in the sciatic nerve of the cat after nerve section. Above, left: mean rate of increase of deoxypentosenucleic acid expressed as μg. P/day/g. wet wt. of nerve (●–●–●). Above, right: mean daily increase of deoxypentosenucleic acid expressed as a percentage of deoxypentosenucleic acid already present (○–○–○). Below, left: mean increase in acid phosphomonoesterase activity expressed as μg. P/hr./μg. deoxypentosenucleic acid P (■–■–■). Below, right: mean increase in β-glucuronidase activity expressed as μg. phenolphthalein/hr./μg. deoxypentosenucleic acid P (□–□–□).

The finding that the activity of β-glucuronidase per unit deoxypentosenucleic acid P increases in degenerating nerves, together with the observations mentioned in the preceding paragraph, could all be brought together in the unified hypothesis of Levvy and his associates that the β-glucuronidase activity increases in conditions associated with a cellular proliferation. In degenerating nerve there is certainly a cellular proliferation and there is also a great increase in β-glucuronidase activity. However, the time relations are difficult to reconcile with such an hypothesis. Fig. 3 shows an attempt to determine the time of maximum cellular proliferation. Two chemical estimates of the degree of proliferative activity have been derived. One is the mean daily increase of deoxypentosenucleic acid expressed as μg. P/g. wet wt. of nerve/day and the other is the mean daily increase of deoxypentosenucleic acid expressed as a percentage of the deoxypentosenucleic acid already present in the nerve. Both estimates show that, whereas the concentration of deoxypentosenucleic acid reached a maximum at 16 days, the peak in the rate of formation of deoxypentosenucleic acid was at 4 days. Fig. 3 also shows that the increase in the activity of the acid phosphomonoesterase (on a deoxypentosenucleic acid basis) is greatest at this same 4-day period, but that the increase in the activity of the β-glucuronidase, on a same basis, is still rising at 16 days.

It thus seems evident that in a degenerating nerve the increase in the β-glucuronidase activity per cell occurs later and persists longer than the cellular proliferation. In this respect the results are similar to those reported by Mills, Smith, Stary & Leslie (1950) and Mills (1951). These workers found that in the liver of the rat, regenerating after subtotal hepatectomy, the peak in the β-glucuronidase activity per unit deoxypentosenucleic acid occurred after the phase of rapid cellular proliferation.

The increase in the activity of β-glucuronidase in degenerating and regenerating nerves is also of interest in relation to the finding of Bernfeld & Fishman (1950) that electrophoretically pure β-glucuronidase from calf spleen was activated by nucleic acid. Deoxypentosenucleic acid was found to be more active than pentosenucleic acid and, subsequently, Bernfeld, Guarino & Fishman (1951) showed that thymidylic acid was active in this respect. As has been noted already, there is a great increase in the concentration of both types of nucleic acid in a degenerating nerve.

SUMMARY

1. The β-glucuronidase activity of the sciatic nerve of the cat increased greatly after either nerve section or nerve crush.

2. After nerve section the increase was statistically significant after 4 days (138%) and reached a maximum at 16 days (3400%). Thereafter it fell steadily.

3. After nerve crush the β-glucuronidase activity did not differ significantly from that observed after nerve section for the first 96 days. Even after 600 days the activity in the crushed nerves was significantly greater than that in the control nerves.

4. The β-glucuronidase activity per cell, calculated on the deoxypentosenucleic acid, increased to a maximum (1120%) at 16 days after nerve section while the peak in cellular proliferation was at 4 days.

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The Inhibition of Erythrocyte Cholinesterase by Tri-esters of Phosphoric Acid

2. DIETHYL *p*-NITROPHENYL THIONPHOSPHATE (E605) AND ANALOGUES

BY W. N. ALDRIDGE AND A. N. DAVISON
Medical Research Council Unit for Research in Toxicology, Serum Institute, Carshalton, Surrey

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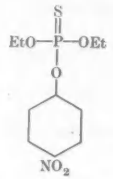
In the first paper of this series (Aldridge & Davison, 1952) it was shown that inhibition of cholinesterase by a series of substituted diethyl phenyl phosphates increased as their stability to hydrolysis decreased. During this work it was found that many of the inhibitors contained a small amount of a more active inhibitor as an impurity and evidence was produced that this was tetraethyl pyrophosphate (TEPP).

Diggle & Gage (1951*a*) have recently shown that a chromatographically purified preparation of diethyl *p*-nitrophenyl thionphosphate (E605) has an extremely low inhibitory power against cholinesterase *in vitro*. In this laboratory this observation has been confirmed using the same sample (Aldridge & Barnes, 1952). However, many workers have stated that E605 is an active inhibitor of cholinesterase *in vitro* (Aldridge, 1950; DuBois, Doull, Salerno & Coon, 1949; Engbaek & Jensen, 1951; Grob, 1950; Hecht & Wirth, 1950; Metcalf & March, 1949; Sallé, 1950; Wirth, 1949). In a report of work carried out during the war years, Schrader (1951) has stated that E605 isomerizes upon distillation and devised a series of tests to determine the diethyl *p*-nitrophenyl thionphosphate content, its purity and freedom from *S*-ethyl isomer. In Table 1 are shown the formulae of the three possible isomers of E605. The whole question of the purity and *in vitro* activity of E605 against cholinesterase has been complicated by the fact that although pure E605 has a low inhibitory power *in vitro* it is still highly toxic to animals and is converted *in vivo* to a more active inhibitor of cholinesterase (Diggle & Gage, 1951*b*; Aldridge & Barnes, 1952). In this paper we have tried to determine the true *in vitro* activity of E605 and its isomers. This has entailed investigation into the purity of our specimens of inhibitors. The specimen of E605 previously used by one of us (Aldridge, 1950) has also been re-examined and it has been found that most of its inhibitory activity is due to diethyl *p*-nitrophenyl phosphate (E600).

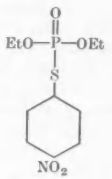
A 'hydrolysis technique' which takes advantage of their different rates of hydrolysis in buffers (Aldridge & Davison, 1952) was developed for the

detection of unstable active inhibitors present as impurities. In the examination of the purity of the inhibitors used in this paper (Table 1) this technique has been considerably extended and, with a method for the determination of inhibitors by their inhibitory power, it has been possible to suggest the identity of impurities. With preparations of thionphosphates whose purity has been established by the methods outlined above, the relation between stability to hydrolysis and *in vitro* inhibitory power has been determined. These additional compounds confirm the relationship previously demonstrated (Aldridge & Davison, 1952).

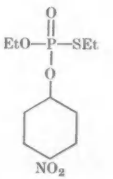
Table 1. *Inhibitors used*



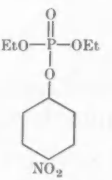
OO-Diethyl *O*-*p*-nitrophenyl thionphosphate (E 605, Parathion, Thiophos)



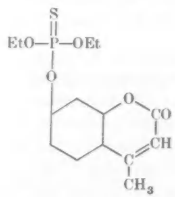
OO-Diethyl *S*-*p*-nitrophenyl thiolphosphate (S-phenyl isomer of E 605)



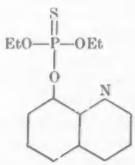
OS-Diethyl *O*-*p*-nitrophenyl thiolphosphate (*S*-ethyl isomer of E 605)



Diethyl *p*-nitrophenyl phosphate (E 600, Paroxon)



OO-Diethyl *O*-7-(4-methylcoumaryl) thionphosphate (E 838, Potasan)



OO-Diethyl *O*-8-quinolyl thionphosphate (Q 2)

METHODS AND MATERIALS

Cholinesterase of sheep erythrocytes has been determined by the manometric method given in detail previously (Aldridge & Davison, 1952).

In the 'hydrolysis technique' the stability of inhibitors has been determined in two buffers. The buffer used for the manometric determination of cholinesterase contains 0·0357 M-$NaHCO_3$, 0·040 M-$MgCl_2$, and 0·164 M-NaCl. This solution has been used for an examination of the hydrolysis of the *S*-ethyl isomer of E 605. When not gassed with 5% CO_2 in N_2 it has a pH of 8–8·5. The hydrolysis of other inhibitors has been studied in a solution at pH 10·8 containing 0·035 M-Na_2CO_3, 0·164 M-NaCl. Rates of hydrolysis of inhibitors in these buffers have been determined chemically by colorimetric estimation of the *p*-nitrophenol or *p*-nitrothiophenol liberated and also biochemically from the inhibitory power of the solution against cholinesterase. The results of such determinations have been plotted logarithmically in the usual manner to test for first-order kinetics and the velocity constants have been calculated. Throughout this paper the stability of inhibitors has been expressed by these constants (cf. Table 3). Diethyl 8-quinolyl thionphosphate (Q 2) was purified chromatographically using alumina (Peter Spence, type H) and a benzene-light petroleum (b.p. 80–100°) mixture in the proportions 1:2. The amount of esterified 8-hydroxyquinoline was determined by hydrolysis in NaOH followed by its estimation colorimetrically by coupling with diazotized sulphanilic acid. The colour was read at 510 mμ.

Rate constants for the reaction of inhibitors with cholinesterase have been calculated from the slope of the best straight line (by the method of least squares) passing through the points obtained when log (% residual activity) is plotted against concentration (M) × time (min.).

We are grateful to Dr A. H. M. Kirby for the pure E 838 and to Mr B. Topley (Albright and Wilson Ltd.) for the rest of the inhibitors. We are particularly grateful to Mr Topley and Dr Coates for the specimen of purified E 605. This sample was very laboriously purified by a combination of a chromatographic procedure and repeated fractional crystallization at low temperatures.

RESULTS

The determination of the concentration of inhibitors. The inhibition of red cell cholinesterase by E 600 and analogues shows the characteristics of a bimolecular reaction with one component in excess:

$$K = \frac{1}{tI} \ln \frac{100}{b},$$

where K = bimolecular rate constant, t = time in min., I = molar concentration of inhibitor and b = percentage residual activity. If the time is maintained constant, a plot of I against $\log_{10} b$ will give a straight line and $\log_{10} 100/b$ may be used as a measure of inhibitor concentrations. Such a measure may be used when studying hydrolyses which have first-order kinetics and absolute concentrations are not required. In Fig. 1 the relationship is shown between the concentration of E 600 and the inhibition of sheep erythrocyte cholinesterase.

A comparison of the rate of loss of inhibitory power against cholinesterase and the rate of hydrolysis of inhibitors determined by chemical means has enabled impurities present in extremely small amounts to be detected (hydrolysis technique). By also using the quantitative method given above it

has been possible to determine the rate of hydrolysis of the impurity and thus to suggest its identity by comparison with the stability of known inhibitors. When the stability of the impurity and the compound are sufficiently different the technique may be used to determine quantitatively highly active and unstable inhibitors in more stable compounds as well as stable impurities in unstable inhibitors.

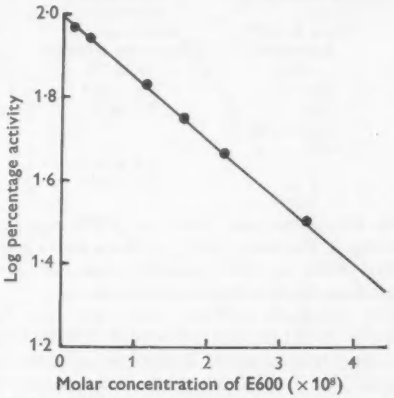


Fig. 1. The inhibition produced by incubation of sheep erythrocyte cholinesterase for 30 min. at 37° with concentrations of E600.

Inhibitory power of two inhibitors together. It has been assumed that the inhibition of cholinesterase by one phosphate ester inhibitor is not affected by the presence of another. In a previous paper (Aldridge & Davison, 1952) the concentration of TEPP in a known mixture with diethyl *p*-chlorophenyl phosphate was determined biochemically from its inhibitory power against cholinesterase under standard conditions. Even though the concentration of diethyl *p*-chlorophenyl phosphate was 150 times that of the TEPP, the determined result was in agreement with the prepared concentration. This can be interpreted as indicating that the inhibition by one inhibitor is not influenced by the other. It was thought desirable to check this point further. The relation between the inhibitory power of inhibitors separately is given by

$$K=\frac{1}{tI}\ln\frac{100}{b} \qquad (1)$$

and

$$K'=\frac{1}{tI'}\ln\frac{100}{b'}. \qquad (2)$$

If these inhibitors do not interfere with the action of each other then from equations (1) and (2)

$$KtI+K'tI'=\ln\frac{100}{b}+\ln\frac{100}{b'}.$$

When t is maintained constant and using for convenience logarithms to the base 10 and K_1 and K_2 for K and K' respectively to account for these changes,

$$K_1I+K_2I'=\log_{10}\frac{100}{b}+\log_{10}\frac{100}{b'}.$$

If the concentrations of inhibitors, I and I', reduce the activity of the cholinesterase after incubation for a standard time to b and b'% of the original activity respectively then the inhibition due to both together may be derived from

$$\log_{10}\frac{100}{b}+\log_{10}\frac{100}{b'}.$$

This relation has been tested using mixtures of diethyl phenyl phosphate and E600. The phenyl compound is present in great excess over the E600, i.e. in one of the experiments 10^{-3}M as compared with 10^{-8}M E600. The results in Table 2 show that the inhibition obtained confirms the relationship derived above and with mixtures of inhibitors of this type little interference of one inhibitor with another is likely.

OO-Diethyl O-p-nitrophenyl thionphosphate, E605. Diggle & Gage (1951*a*) state that the activity of a series of samples of E605 as inhibitors of cholinesterase paralleled their content of *S*-ethyl isomer of E605. Clearly, the assessment of these results

Table 2. *The inhibition of cholinesterase by two inhibitors together*

(E600 and diethyl phenyl phosphate were incubated separately and together with washed sheep erythrocytes for 30 min. at 37°. The cholinesterase activity remaining was determined. The concentrations used were for Exp. 1, E600 2×10^{-8}M, diethyl phenyl phosphate $9{\cdot}8\times10^{-4}$M; for Exp. 2, E600 2×10^{-8}M, diethyl phenyl phosphate $8{\cdot}4\times10^{-2}$M; and for Exp. 3, E600 $1{\cdot}25\times10^{-8}$M, diethyl phenyl phosphate $4{\cdot}0\times10^{-2}$M. b = % residual activity.)

	Diethyl phenyl phosphate		E600		Diethyl phenyl phosphate + E600			
					Determined values		Calculated values	
Exp. no.	Activity (%)	(1) $\log_{10}\frac{100}{b}$	Activity (%)	(2) $\log_{10}\frac{100}{b}$	Activity (%)	$\log_{10}\frac{100}{b}$	Activity (%)	(1)+(2) $\log_{10}\frac{100}{b}$
1	83·4	0·079	48·1	0·318	41·7	0·380	40·1	0·397
2	21·0	0·678	46·9	0·329	12·0	0·921	10·1	1·007
3	47·5	0·323	66·2	0·179	31·1	0·507	31·7	0·502

Table 3. *The rate of hydrolysis of inhibitors in sodium carbonate at* pH 10·8 *and* 37°

(The rate of hydrolysis of inhibitor has been determined chemically by the estimation of *p*-nitrophenol and *p*-nitrothiophenol liberated, or biochemically by the determination of the inhibitory power of the solution against sheep erythrocyte cholinesterase (see text, p. 664). All hydrolysis rates were determined in a solution containing Na_2CO_3 (0·035M), NaCl (0·164M), except the *S*-ethyl isomer which was hydrolysed too quickly in this medium. The expected rate at pH 10·8 was calculated from values at pH 7·6 (0·067M-phosphate) and pH 9·64 (Michaelis veronal-acetate) using the expression $K = K_{H_2O} + K_{OH}[OH]$ and neglecting any catalysis by $(H_3O)^+$ ions.)

	Hydrolysis determined chemically		Hydrolysis determined biochemically
Inhibitor	First-order constant (min.$^{-1}$)	Time for 95% hydrolysis (hr.)	First-order constant (min.$^{-1}$)
E600	$2·7 \times 10^{-3}$	18·5	$2·9 \times 10^{-3}$
E605	$4·1 \times 10^{-4}$	122	3×10^{-4}
S-ethyl isomer	5×10^{-1}	Approx. 0·1	—
S-phenyl isomer	$9·6 \times 10^{-2}$	0·53	—
Impurity in *S*-ethyl isomer	—	—	$3·1 \times 10^{-3}$
Impurity in E605	—	—	$2·5 \times 10^{-3}$

depends on the specificity of the method of estimation (Gage, 1952) of this isomer in complex mixtures. It will be shown later that the specimen of purified *S*-ethyl isomer used by Diggle & Gage contained roughly 4% of E600. The chemical structure of E605 makes it unlikely that it would have no inhibitory activity against cholinesterase. It was, therefore, decided to see if pure E605 had any activity or whether the low activity of the purest sample available is also due to a trace of the *S*-ethyl isomer. Because of the extreme instability of the *S*-ethyl isomer in aqueous solution compared with E605 (cf. Tables 3 and 4) it was possible to investigate this point. A solution of the sample of purified E605 was prepared in sodium carbonate solution (pH 10·8). Under these conditions 95% of any *S*-ethyl isomer present would be hydrolysed in 6 min., the *S*-phenyl isomer in 32 min. and E600 in 18 hr. (Table 3). When the inhibitory power of the solution was determined at various times it was found that there was 75% of the activity left at 20 hr. It is clear that the inhibitory power cannot be due to any of the above three inhibitors. The activity of the solution fell exponentially and the rate constant in Table 3 shows that the activity falls at roughly the same speed as the hydrolysis of E605 determined chemically. In view of the low solubility of E605 and the low inhibitory power of the saturated solution this is as good an agreement as can be expected, and indicates that the anticholinesterase activity of the solution is probably due to E605 itself.

The first-order rate constant for the hydrolysis of E605 has been determined in phosphate buffer, pH 7·6, at 37°, one of the hydrolysis products, *p*-nitrophenol, being estimated colorimetrically. The rate constant for the reaction of E605 with erythrocyte cholinesterase has also been determined (Table 4). In Fig. 2 these values are plotted, and it is interesting that this point falls near the line established from previous work on E600 analogues (Aldridge & Davison, 1952), i.e. the activity of this purified E605 is approximately what one would expect from its stability to hydrolysis.

E605 previously studied. Previously, one of us (Aldridge, 1950) studied a specimen of E605 which must have been impure. First-order kinetics were obtained for its reaction with cholinesterase and the rate constant for this reaction was 2×10^4 (l. mole^{-1} min.$^{-1}$), whereas it has now been shown that a purified E605 gives a rate constant of $1·2 \times 10^2$ (l. mole^{-1} min.$^{-1}$). Diggle & Gage (1951*a*) have demonstrated a relation between the *S*-ethyl isomer content of various specimens of E605 and their inhibitory activity. As will be shown later, the inhibition of cholinesterase by *S*-ethyl isomer does not follow first-order kinetics. It therefore seemed improbable that *S*-ethyl isomer was responsible for the activity, and an examination of this specimen of E605 has been made. Its inhibitory power has remained unaltered since it was first examined and at a concentration of $1·3 \times 10^{-6}$M it inhibits sheep erythrocyte cholinesterase 50% after incubation for 30 min. at 37°. The rate of loss of inhibitory power of a solution of this inhibitor in sodium carbonate, pH 10·8, has been determined. The rate constant for this hydrolysis is given in Table 3, and indicates that the impurity cannot be the *S*-ethyl or the *S*-phenyl isomer and is in all probability E600. 1·6% E600 would account for the inhibitory power of this specimen of E605.

OO-*Diethyl* S-p-*nitrophenyl thiolphosphate* (S-*phenyl isomer*). This isomer is a pale yellow crystalline solid and is therefore much more readily purified than a liquid with a high boiling point. It is a highly active inhibitor producing 50% inhibition at 37° in 30 min. at a concentration of $2·8 \times 10^{-8}$M. The graph of $\log_{10}$ residual activity against the time of incubation of inhibitor with cholinesterase shows that the lines are slightly curved (Fig. 3). This can be

accounted for by the expected loss of inhibitor due to aqueous (non-enzymic) hydrolysis alone. In phosphate buffer, pH 7·6, at 37° the first-order hydrolysis constant is $5{\cdot}3 \times 10^{-4}$ (min.$^{-1}$); in 2 hr. therefore 7% of the inhibitor would have been destroyed by this means.

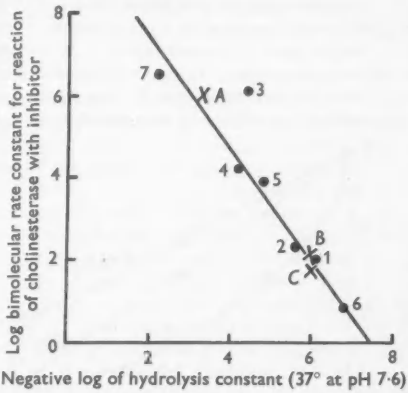


Fig. 2. The relation between stability to hydrolysis and inhibitory power. ●, points taken from Aldridge & Davison (1952). ×, compounds studied in this paper. 1–7 derivatives of diethyl phosphoric acid: 1, *p*-chlorophenyl; 2, *o*-chlorophenyl; 3, *p*-nitrophenyl (E600); 4, *o*-nitrophenyl; 5, *m*-nitrophenyl; 6, phenyl; 7, tetraethyl pyrophosphate. *A*, *S*-phenyl isomer of E605; *B*, E605; *C*, Q2.

Table 4. *Velocity constants for the reaction of inhibitors with cholinesterase and for their hydrolysis in phosphate buffer*, pH 7·6

(Rates of hydrolysis were determined colorimetrically by the estimation of *p*-nitrophenol or *p*-nitrothiophenol liberated for all inhibitors except Q2. A rough value for Q2 has been determined by calculation from the loss of inhibitory power at 37° in Na_2CO_3 solution, pH 10·8 (Na_2CO_3 (0·035 M), NaCl (0·164 M)) and in a $NaHCO_3/Na_2CO_3$ solution, pH 8·96 (Na_2CO_3 (0·0037 M), $NaHCO_3$ (0·0238 M), NaCl (0·164 M)) using the expression $K = K_{H_2O} + K_{OH}[OH]$ and neglecting any catalysis by $(H_3O)^+$ ions. The velocity constants for reaction with cholinesterase were calculated from the slope of the best straight line obtained on plotting concentration of inhibitor (M) × time (min.) against ln % activity of cholinesterase.)

Inhibitor	Rate constant (K) for reaction of inhibitor with cholinesterase (l.mole^{-1} min.$^{-1}$)	Rate constant (K) for hydrolysis of inhibitor at pH 7·6 in phosphate buffer (min.$^{-1}$)
E600	$1{\cdot}1 \times 10^{6}$	$3{\cdot}9 \times 10^{-5}$
E605	$1{\cdot}2 \times 10^{2}$	$9{\cdot}6 \times 10^{-7}$
S-Ethyl isomer of E605	—	$1{\cdot}1 \times 10^{-3}$
S-Phenyl isomer of E605	$9{\cdot}7 \times 10^{5}$	$5{\cdot}3 \times 10^{-4}$
Q2	$4{\cdot}6 \times 10^{1}$	Approx. 1×10^{-6}
E838	$1{\cdot}5 \times 10^{2}$	—

The constants obtained for this inhibitor fit the relationship between rate of reaction with cholinesterase and stability to hydrolysis (Fig. 2).

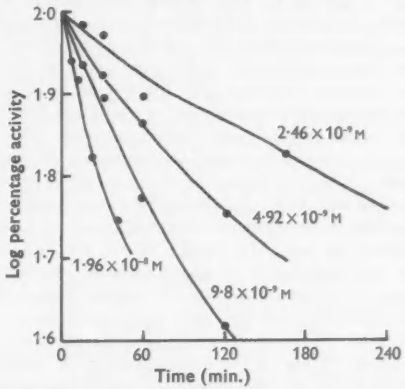


Fig. 3. The inhibition of sheep erythrocyte cholinesterase by the *S*-phenyl isomer of E605. Concentrations of *S*-phenyl isomer shown against each curve.

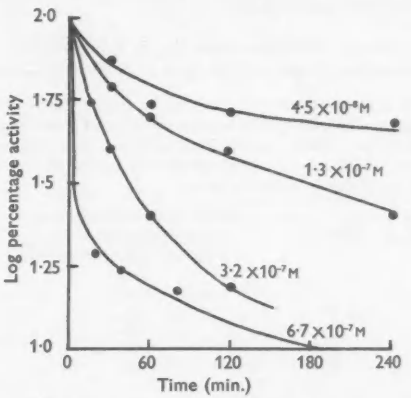


Fig. 4. Inhibition of sheep erythrocyte cholinesterase by *S*-ethyl isomer of E605 (impure). Concentration of *S*-ethyl isomer shown against each curve.

OS-*Diethyl* O-p-*nitrophenyl thiolphosphate* (S-*ethyl isomer*). The rate of reaction of this inhibitor with cholinesterase was determined at several concentrations. Fig. 4 shows that a series of curves was obtained when log (% residual activity) was plotted against time. These results resembled those obtained with the analogues of E600 which were found to be contaminated with TEPP (Aldridge & Davison, 1952). TEPP is destroyed by sheep erythrocytes. An initial rapid inhibition due to the TEPP was obtained, but after 20–30 min. this had all been destroyed and the residual rate was due to

the E600 analogue being examined. The *S*-ethyl isomer is unstable in water and the first-order rate constant at pH 7·6 for its hydrolysis is $1{\cdot}1 \times 10^{-3}$ (min.$^{-1}$) (Table 4). This means that during the 240 min. incubation of inhibitor with cholinesterase (Fig. 4) approximately 22% of the inhibitor would have been hydrolysed. Although sheep erythrocytes do not catalyse the hydrolysis of E600, experiments were carried out to see if the *S*-ethyl isomer was hydrolysed. When *S*-ethyl isomer (approx. 1 mg./ml.) was incubated in Warburg vessels, little difference in output of CO_2 was observed with and without erythrocytes. This indicates that the hydrolysis of *S*-ethyl isomer by sheep erythrocytes must be small. When erythrocytes were incubated with a dilute solution of *S*-ethyl isomer, samples withdrawn at various times, the cells centrifuged down and the supernatant re-incubated with fresh erythrocytes, no loss of inhibitory power of the solution could be demonstrated (Table 5). This experiment should assess the total loss of *S*-ethyl isomer due to both non-enzymic and enzymic hydrolysis. It is clear that there was in the solution an inhibitor which was not destroyed under these conditions.

Table 5. *Inhibitor remaining in solution after incubation of sheep erythrocytes with* S*-ethyl isomer*

(*S*-Ethyl isomer, $5{\cdot}6 \times 10^{-7}$ M, was incubated with washed sheep erythrocytes. The erythrocytes were centrifuged down after various times of incubation and the inhibitory power of the supernatant was determined by incubation with fresh erythrocytes for 30 min.)

Time of incubation (min.)	Activity of cholinesterase of erythrocytes after incubation with supernatant (%)
0	20
10	21
20	20
40	20
90	18

The observation that the rate of inhibition decreased with time (Fig. 4) suggested that an equilibrium was being reached between enzyme and inhibitor. If this were so it would be expected that the inhibition, like that due to eserine, would be reversible. Repeated washing of erythrocytes after 4 hr. incubation with inhibitor gave no increase in enzyme activity, however.

These observations differ from our previous experience with inhibitors of this type. Pure substituted diethyl phenyl phosphates all give first-order kinetics and the rate is directly proportional to the concentration of inhibitor. However, abnormal kinetics were obtained when the original unpurified inhibitors contained as an impurity the highly active and unstable TEPP. It was therefore important to consider the purity of our specimen of *S*-ethyl isomer.

There is no specific chemical method for the determination of *S*-ethyl isomer, and evidence of purity must be deduced from other data. Topley (personal communication) has carried out detailed kinetic measurements on the hydroxyl ion-catalysed hydrolysis of this specimen of *S*-ethyl isomer. First-order kinetics were obtained with no detectable drift in the rate constant. It was concluded that the compound is at least 90% pure. The measurements we have made from which the rate constant given in

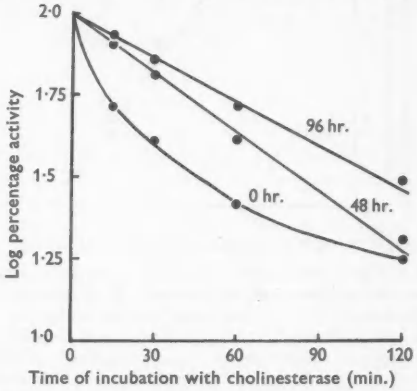


Fig. 5. Inhibitory activity of $2{\cdot}24 \times 10^{-6}$ M-*S*-ethyl isomer of E 605 in bicarbonate buffer, pH 8·4, after incubation at 37°. Time of incubation shown against each curve. The solution was diluted seven times for incubation with cholinesterase to determine inhibitory power.

Table 4 was calculated confirm this view, and elementary analysis is not at variance with it. However, when hydrolysis of the isomer was followed in bicarbonate buffer, pH 8·4, at 37°, estimating the inhibitory power of the solution against cholinesterase, results were obtained which did not agree with chemical measurements. When the isomer had decomposed (as shown by the liberation of *p*-nitrophenol) the solution still possessed most of its inhibitory power (Fig. 5). In contrast to the initial results, the rate of inhibition of cholinesterase by the solution after 48 hr. incubation follows first-order kinetics. It was deduced from these observations that our *S*-ethyl isomer contained as an impurity an active inhibitor of cholinesterase which is considerably more stable than *S*-ethyl isomer. An attempt was therefore made to identify this impurity. When a solution of the isomer which has been left for 7 days in bicarbonate buffer at room temperature is extracted with chloroform, washed free from *p*-nitrophenol with bicarbonate, the chloroform evaporated and the residue taken up in buffer, the resulting solution

inhibited cholinesterase and the rate of inhibition followed first-order kinetics. This solution, which lost its activity slowly over the following weeks, became pale yellow. The yellow colour was extracted with *n*-butanol from acid solution and then transferred back to a small volume of buffer. The visible and ultraviolet absorption spectra of this solution, both acid and alkaline, were identical with that of *p*-nitrophenol.

The impurity present is more stable than the *S*-ethyl isomer and liberates *p*-nitrophenol upon hydrolysis. Diethyl *p*-nitrophenyl phosphate (E600) is a highly active inhibitor which fulfils these conditions. This possibility has been examined. It has been shown that the inhibitory power of a solution of the isomer prepared 1 hr. previously in sodium carbonate solution, pH 10·8, falls at the same rate as that of a solution of E600. This value agrees with the rate of hydrolysis of E600 determined chemically (Table 3). In sodium carbonate buffer, pH 10·8, and at 37° the *S*-ethyl isomer itself will be 95% hydrolysed in 6 min. and any *S*-phenyl isomer in 32 min. It is concluded, therefore, that the impurity is E600. An estimate of the amount of E600 present may be obtained from the curves given in Fig. 5 on the assumption that all the activity at 48 and 96 hr. is due to E600. The concentrations of E600 necessary to produce this inhibition are calculated from the known bimolecular rate constant ($1{\cdot}1 \times 10^6$ l.mole^{-1} min.$^{-1}$) and this is then corrected for aqueous hydrolysis back to zero time. By this means we obtain 4·5 and 4·1% from the results after incubation for 48 and 96 hr. respectively.

OO - *Diethyl* O - 7 - (4 - *methylcoumaryl*)*thionphosphate* (*E*838). This inhibitor which is crystalline (m.p. 41·5°) shows little activity *in vitro* against erythrocyte cholinesterase. A saturated solution contains approximately 5 μg./ml. and at this concentration ($1{\cdot}3 \times 10^{-5}$ M) only 54% inhibition is produced in 5 hr. No loss of activity of a solution of this compound occurred after incubation at 37° for 14 days at pH 8. It is, therefore, concluded that this sample of inhibitor did not contain any unstable active inhibitor as an impurity. Because of its low solubility and activity the data on the kinetics of inhibition are limited. Sufficient has been obtained to suggest that first-order kinetics are obtained and on the assumption that it is bimolecular the rate constant has been calculated (Table 4). Due to the lack of a method of high sensitivity for the determination of 7-hydroxy-4-methylcoumarin we have been unable to determine the rate of hydrolysis of E838.

OO - *Diethyl* O - 8 - *quinolyl thionphosphate* (*Q*2). Aldridge (1950) showed that inhibition by this substance followed first-order kinetics, but the straight line obtained when $\log_{10}$% activity was plotted against time did not pass through the zero. Part of this inhibition was reversible and the amount below the zero where the lines cut the ordinate [2−($\log_{10}$ % activity)] was a measure of this reversible inhibition. In view of the impurities in other inhibitors derived from thiophosphoric acid this compound has been re-examined. A solution of Q2 in a 1:2 benzene-light petroleum mixture was passed through an alumina column. A preparation of Q2 was obtained which contained the theoretical amount of combined 8-hydroxyquinoline. When the inhibitory power of this purified sample was examined, first-order kinetics were obtained (Fig. 6) and the straight lines passed through the zero (100% activity).

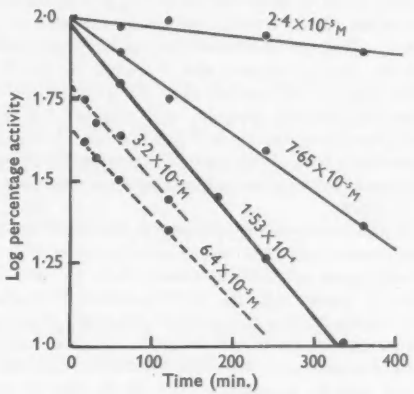


Fig. 6. Inhibition of sheep erythrocyte cholinesterase by Q2. Concentrations of inhibitor shown against each curve. ——, purified as in text; - - - -, unpurified sample as used by Aldridge (1950).

Further determinations with the unpurified sample confirmed the original observations of Aldridge (1950), but the material(s) responsible for the reversible inhibition have not been identified. Since Q2 is only soluble to approx. 40 μg./ml. long incubation times have been used in order to obtain reasonable inhibitions. The reaction appears to be bimolecular and the rate constant has been determined from the limited data available (Table 4).

DISCUSSION

Many investigators have shown that E605 is an *in vitro* inhibitor of cholinesterase. Estimates of the inhibitory power of different samples have varied, but until the observations of Diggle & Gage (1951*a*) it was not necessary to consider the possibility that E605 is converted *in vivo* to a more active inhibitor of cholinesterase. Evidence has been produced that this is so (Diggle & Gage, 1951*b*; Aldridge & Barnes, 1952). Diggle & Gage (1951*a*) have concluded that the inhibitory power of the specimens of E605 they

examined was due to their content of *S*-ethyl isomer. However, Gage (1952) states that using his methods of analysis the highly active inhibitors, *S*-phenyl isomer and E600, will both behave like the relatively inactive E605 and will be reported as such. Evidence has been presented which indicates that pure E605 has an *in vitro* inhibitory activity of its own. The *S*-ethyl isomer studied in this paper is from the same preparation that Diggle & Gage (1951*a*) used as their standard. We have shown that it contains approximately 4% E600. The high inhibitory activity of a specimen of E605 used previously by one of us (W.N.A.) has been explained by the presence of 1·6% E600. It is clear that caution must be observed before any generalizations are made about the nature of the impurities in various E605 preparations. The known possibilities are the *S*-ethyl isomer, the *S*-phenyl isomer and E600, and it is probable that the proportions of these substances present will depend on the methods of preparation and purification. It is clear from the reports of the work of Schrader (1951) that any form of distillation is inadvisable as a method of purification.

In a previous paper (Aldridge & Davison, 1952) it was demonstrated that as the stability to hydrolysis of analogues of E600 increased their *in vitro* inhibitory power against cholinesterase decreased. The results in this paper show that the *S*-phenyl isomer fits well into this relation and the limited data obtained with E605 and Q2 show that there is no reason to doubt that they also fit the same relation. The hydrolysis constant for E838 could not be determined either by chemical or biochemical methods. The *S*-ethyl isomer does not have the inhibitory activity expected from its stability to hydrolysis. Its first-order hydrolysis constant is $1{\cdot}1 \times 10^{-3}$ (min.$^{-1}$) in phosphate buffer, pH 7·6, at 37°. From the relation shown in Fig. 2, *S*-ethyl isomer should have a bimolecular rate constant for its reaction with cholinesterase of about 10^6 (l.mole^{-1} min.$^{-1}$). This value corresponds to 50% inhibition after incubation for 30 min. at 37° with a concentration of 2×10^{-8}M. It is clear from Fig. 4 that 50% inhibition is produced by approximately 3×10^{-7}M and this is uncorrected for the inhibitory power of the 4% E600 present in this specimen of isomer. This isomer does not give the usual first-order kinetics on reaction with cholinesterase, but until we have obtained a purer specimen of this inhibitor any suggestions of reasons for such behaviour would be speculative. It is interesting that Kilby & Youatt (1952) have recently shown that, acting on trypsin, the *S*-ethyl isomer is a more potent inhibitor than E600.

Many organo-phosphorus compounds are being prepared by research organisations. Traces of impurities may profoundly alter their biological activity. Without further evidence statements of inhibitory power (as concentrations to produce 50% inhibition) must be regarded as only applying to the particular specimen of inhibitor which has been examined. The 'hydrolysis technique' we have developed for testing inhibitors for small amounts of impurities is based on the determination of the concentration of an inhibitor by its inhibitory power. It is, therefore, possible to compare the rate of hydrolysis of the main constituent determined by chemical methods and the rate of loss of inhibitory power determined biochemically. When these do not agree impurities should be suspected and often these may be identified from their hydrolysis rates under standard conditions. These tests will detect unstable impurities in stable inhibitors (TEPP in analogues of E600) and stable impurities in unstable inhibitors (E600 in *S*-ethyl isomer). It is not possible to detect impurities of a similar stability. Many impurities may be eliminated by rigorous purification of the materials used for synthesis but others may arise through side reactions. Traces of impurities in liquids with high boiling points may be extremely difficult to remove by the conventional chemical techniques. The purification achieved can, at present, only be assessed by a biochemical examination.

Bimolecular rate constants are a better measure of inhibitory power than the conventional concentration for 50% inhibition. An examination of the kinetics of inhibition will often indicate the presence of impurities. These will remain undetected with a simple determination of 50% inhibition concentration. TEPP which is at least 90% hydrolysed in 25 min. by sheep erythrocytes gives an apparently normal inhibition curve on plotting $\log_{10}$M concentration of inhibitor against percentage inhibition. In all cases except one (*S*-ethyl isomer) abnormal kinetics of inhibition have been shown to be due to impurities, or to aqueous and enzymic hydrolysis of the inhibitor or a combination of both factors. However, if first-order kinetics are obtained it does not necessarily mean that the sample is pure. This is quite clear from the results on a specimen of E605 containing 1·6% E600 (Aldridge, 1950).

In conclusion, it is suggested that an examination of the kinetics of inhibition of erythrocyte cholinesterase together with the application of the 'hydrolysis technique' will help in the detection and sometimes the determination of active impurities in organo-phosphorus inhibitors. The converse is that before it is stated that the reaction of an inhibitor with erythrocyte cholinesterase does not follow first-order kinetics, it must be demonstrated that the inhibitor is not appreciably hydrolysed during the experiment either enzymically or by aqueous hydrolysis and also that it does not contain highly active impurities.

SUMMARY

1. Pure E605 has a low *in vitro* activity against cholinesterase.

2. A specimen of E605 previously used (Aldridge, 1950) owed its high activity to the presence of E600.

3. The *S*-phenyl isomer of E605 is a highly active inhibitor and the inhibition follows first-order kinetics. The specimen of *S*-ethyl isomer contains approximately 4% E600.

4. It was observed (Aldridge & Davison, 1952) that in a series of diethyl phenyl phosphates substituted in the aromatic ring, the more stable to hydrolysis the inhibitor the less effective it is as an inhibitor. This relationship has been extended to include E605, Q2 and the *S*-phenyl isomer of E605.

5. A 'hydrolysis technique' has been developed and kinetic measurements have been utilized for the detection and determination of impurities in organophosphorus inhibitors. These methods are fully discussed.

Our thanks are due to Miss J. Wheatley for valuable technical assistance.

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Amylases of *Clostridium butyricum* and a *Streptococcus* Isolated from the Rumen of the Sheep

By P. N. HOBSON and MARGARET MACPHERSON
The Rowett Research Institute, Bucksburn, Aberdeenshire

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In the course of an investigation into the digestion of starch by the pig, Baker, Nasr, Morrice & Bruce (1951) isolated a micro-organism responsible for the breakdown of starch in the caecum and identified it as a strain of *Clostridium butyricum*. Later Masson (1951) isolated another strain of *Cl. butyricum* which was the chief agent of starch breakdown in the rumen of a sheep fed on a flaked maize and hay diet. Continuing these investigations into the digestion of starches by the sheep, MacPherson (forthcoming publication) obtained several strains of a *Streptococcus*, probably related to *Strep. bovis*, from the rumen, one being selected for use throughout this work. The extracellular amylase formed when the pig caecum strain of *Cl. butyricum* was grown on a medium containing soluble starch was examined by Whelan & Nasr (1951) and was shown to be of the α-amylase type. Both the sheep rumen *Clostridium* and *Streptococcus* formed an extracellular amylase when grown in a medium containing dissolved starch, and an investigation of these amylases is the subject of this paper. The amylases would appear to be constitutive and not adaptive enzymes since, when the *Clostridium* was grown in a medium containing maltose as the only carbohydrate, and the *Streptococcus* on a glucose-containing medium, the other constituents being the same as in the starch medium, amylase activity was present in the cell-free filtrates.

There are at present known to be two major groups of starch-hydrolysing enzymes, the α- and β-amylases. In addition, there is the amylase of *Bacillus macerans* which has a synthetic as well as a hydrolytic function, as it catalyses the synthesis of cyclic 'Schardinger dextrins' from starch and starch fractions. Recently, also, various authors have reported the isolation of a maltase from a bacterium and moulds which is also capable of hydrolysing starch entirely to glucose (French & Knapp, 1950; Kerr, Cleveland & Katzbeck, 1951; Philips & Caldwell, 1951*a*, *b*). The only sugar produced during the hydrolysis of starch-type polysaccharides by β-amylase, which is found in ungerminated cereals, is maltose. Until recently it was thought that amylose, the 'linear' component of starch, was entirely

converted to maltose by this enzyme, but recent work by Peat, Pirt & Whelan (1952) has shown that the purest samples of β-amylase produce only 70% of the theoretical maltose from amylose. The presence of a second enzyme (Z-enzyme), usually present in β-amylase preparations, and which has been shown to be a β-glucosidase (Peat, Thomas & Whelan, 1952), is needed to enable the hydrolytic action of the β-amylase to continue beyond this arrest point. β-Amylase degrades the polysaccharide chain from the non-reducing end group and its action is arrested by the presence of the α-1:6-linkages in amylopectin, the 'branched-chain' component of starch, leading to the formation of a limit dextrin, 'β-dextrin'. The α-amylases on the other hand degrade starch and starch fractions in a more random manner and can hydrolyse the α-1:4-linkages of the inner chains of amylopectin between the α-1:6-branch linkages forming first dextrins and then a mixture of reducing sugars. This property of the α-amylases is generally used for testing for their presence in β-amylase preparations. Pure β-amylase does not attack β-dextrin, but a small concentration of α-amylase will hydrolyse α-1:4-linkages beyond the branch points, thus exposing more non-reducing end groups to the action of the β-amylase and resulting in degradation of the dextrin. The presence of β-amylase in α-amylase preparations is more difficult to detect, but Hobson, Whelan & Peat (1950) have recently introduced a method for characterizing α-amylase by comparing its actions on amylose and β-dextrin and this has been applied to the amylases described in this paper. Quantitative determinations of amylase activity depend on the measurement of either the reducing sugars formed, or the change in iodine stain of starch fractions under standard conditions. It is usual to measure the maltose formed by β-amylase, while, since the reducing power of the initial products of α-amylolysis is small, α-amylase activity is generally determined by observing the time taken for a starch digest to attain a standard iodine colour, or by measuring the change in iodine colour of the digest over a standard time. It should be emphasized, however, that the presence of one amylase as impurity can lead to erroneous results in measuring the activity of the other.

The bacterial amylases so far studied have, with the exception of the *B. macerans* enzyme, been of the α-type. The amylase of *B. subtilis* was investigated by Hopkins & Kulka (1942), and Hopkins, Dolby & Stopher (1942), and by Di Carlo & Redfern (1947*a*, *b*, 1948). This enzyme has now been obtained in a crystalline form by Meyer, Fuld & Bernfeld (1947) and in its saccharifying and dextrinizing action on starch it appears to be similar to salivary, pancreatic and malt α-amylases, but the nature of the end products of the hydrolysis have not been reported. Hopkins *et al.* (1942), however, showed that the *B. subtilis* enzyme was an α-amylase and that it was similar to malt α-amylase in that it rapidly hydrolysed starch until the sugars liberated corresponded to 40% apparent maltose. Thereafter the rate of hydrolysis was slower until a limiting conversion of 75–80% apparent maltose was found. Rose (1948), investigating the properties of an amylase formed by *B. polymyxa*, found that the reducing power of the products of the hydrolysis of starch by this enzyme did not exceed 80% theoretical maltose. Hockenhull & Herbert (1945) were not able to obtain a maltase-free enzyme from *Cl. acetobutylicum* and, although the rate of hydrolysis decreased when about 70% apparent maltose had been liberated, conversions of starch to considerably greater values than 100% apparent maltose were observed. On the basis of 100% conversion of starch to glucose by the maltase-containing enzyme it was concluded that the α-amylase hydrolysed starch completely to maltose. However, the recent work of French & Knapp (1950) renders this conclusion invalid as they have shown that the maltase of *Cl. acetobutylicum* alone converts starch and dextrins entirely to glucose. More recently, Whelan & Nasr (1951) described the properties of the amylase of a strain of *Cl. butyricum* isolated from the pig caecum. The enzyme was an α-amylase which rapidly degraded potato starch and amylose until an apparent conversion to maltose of 65–66% was reached. Thereafter the hydrolysis ceased.

METHODS AND MATERIALS

Determination of reducing sugars. The copper reagent of Somogyi (1945*a*) standardized against maltose solution was used. Before being added to the reagent portions of the digests containing the *Cl. butyricum* amylase preparations were neutralized with a predetermined volume of 1 N-NaOH and deproteinized with $ZnSO_4$ and $Ba(OH)_2$ as described by Somogyi (1945*b*) as the enzyme preparation was, otherwise, found to lower the apparent maltose concentration. Since the streptococcal amylase preparations had only a slight effect on the reagent it was found more convenient to standardize the Somogyi reagent against maltose solutions containing the enzyme preparation in the concentrations of the digests and to determine the reducing power of the digest samples from these standard graphs, after neutralization with a predetermined volume of 1 N-NaOH. The iodometric technique was used throughout.

Measurement of the iodine stain of polysaccharides. (1) *Blue value* (*BV*). The blue value of a polysaccharide is defined as the reading on the logarithmic scale of a Spekker photoelectric absorptiometer (Hilger and Watts, London), when the absorption of light (relative to water) by a solution of a polysaccharide-iodine complex contained in a 4 cm. cell and having the composition, polysaccharide (1 mg.), I_2 (2 mg.), KI (20 mg.)/100 ml. is measured using Ilford gelatin filter 608 transmitting light of wavelength 680 mμ. (see Bourne, Haworth, Macey & Peat, 1948). The blue value is thus a characteristic of the starch fraction. (2) *EEL blue value* (*EBV*). In this work the light absorption of the polysaccharide-iodine solutions of the concentrations given above contained in a standard $\frac{5}{8}$ in. diameter colorimeter tube was measured in an 'EEL' colorimeter (Evans Electroselenium Ltd., Harlow, Essex) using filter 205 transmitting light of wavelength 680 mμ. A reading of 51·2 on the EEL scale is equivalent to a Spekker reading of 1·40. Thus 1 *EBV* is equivalent to 36·6 *BV*. (3) *Absorption value.* (*AV*). The absorption value is a measurement of the iodine stain of a polysaccharide solution made in the same way as a blue value except that the wavelength of the light used and the polysaccharide concentration are not standard. The wavelength of light used is stated whenever an absorption value is quoted, but the concentration of polysaccharide may be unknown, as, for example, during an amylolysis. (4) '*EEL*' *value* (*EV*). This is an absorption value measured on the EEL colorimeter instead of a Spekker absorptiometer.

Fractionation of potato starch. Amylose was obtained from potato starch by the $Al(OH)_3$-thymol method of Hobson, Pirt, Whelan & Peat (1951). Amylopectin was prepared by fractionation of thymol-amylopectin with methanol as described by these authors. All starch fractions were dried *in vacuo* at 60° over P_2O_5 before use.

Preparation of β-dextrin. This was prepared by the hydrolysis of thymol-amylopectin with soya-bean β-amylase as described by Hobson, Whelan & Peat (1950).

Measurement of α-amylase activity. A colorimetric method was used for determination of the amylase activity, as this was found to need less enzyme than the method previously described where the reducing power of an amylase-starch digest was measured after incubation under standard conditions (Hobson, quoted by Whelan & Nasr, 1951). β-Dextrin was used in preliminary experiments, but it was found that the fall in *EV* (680 mμ.) was proportional to the amount of enzyme present only over a small range, so amylose, of blue value 1·2, was substituted for the dextrin. The amylose (approx. 50 mg.) was dissolved in 0·1 N-NaOH (10 ml.) by heating in a boiling-water bath for not more than 9 min. After cooling, the solution was made just acid to phenolphthalein with 5 N-H_2SO_4 and appropriately diluted. The volume added to the digest mixture was arranged to bring the final concentration of amylose in the digest to 1 mg./ml. The standard digest mixture contained 3·75 ml. amylose, 1·5 ml. of 0·2 M-sodium citrate buffer, pH 7·0, and enzyme solution (1·75 ml.), the digest contents being preheated to 35° before mixing. The digest was incubated at 35° for 30 min. when 1 ml. was removed and stained with I_2 (2 mg.), KI (20 mg.)/100 ml. water for measurement of the *EV* (680 mμ.). This *EV* (680 mμ.) was compared with that of a similar portion of a blank digest containing water in place of the enzyme, and, providing the fall in *EV* (680 mμ.) was not more than 24 units on the colorimeter scale it was strictly proportional to the amount of enzyme present. One activity unit is defined as the amount of amylase which will produce a fall in *EV* (680 mμ.) of one colorimeter scale unit under the above conditions.

Standard digests for determination of the action of the enzyme preparations on polysaccharides. Solutions of starch or starch fractions were prepared as above and incorporated in digests containing 7·5 ml. polysaccharide solution (14 mg.), 0·2 M-sodium acetate buffer of the appropriate pH value (3·0 ml.), water (1·5 ml.), and enzyme solution (2 ml.; activity 66 units). The digest was incubated at 35° and at intervals portions (usually 3 or 5 ml.) were removed for determination of the reducing power, against portions of a blank digest, containing water in place of the polysaccharide solution, which was incubated in parallel. Where necessary the digest volumes were increased to enable more determinations to be made.

Human salivary α-amylase. Saliva was collected as needed, diluted with an equal volume of water and centrifuged to remove the mucin. A portion of the supernatant liquid was then diluted to give a solution of the required activity.

Preparation of cell-free filtrates. (1) *Cl. butyricum.* Cultures of the *Clostridium* were inoculated into a modified Beijerinck fluid medium (400 ml.) containing 5% soluble starch (Baker *et al.* 1951) and incubated at 40° for 40 hr. After this time all iodine-staining polysaccharide in the medium had been degraded and gas production had almost ceased. The culture was centrifuged for 30 min. at 740 *g* to remove most of the cells, and then passed through a Seitz filter fitted with a Carlson 'EK' sterilizing pad. The volume of sterile filtrate obtained was usually about 300 ml. (2) *Streptococcus.* The medium used for these organisms was a yeast broth (200 ml.; 1% 'Difco' yeast extract, 1% Bacto Tryptone, 0·1% KH_2PO_4, pH adjusted to 6·8) containing 5% dissolved starch and 3% (w/v) $CaCO_3$. The culture was incubated at 40° for 40 hr. with occasional shaking to suspend the $CaCO_3$. At this time amylase activity was maximal. The culture was then centrifuged until clear (20 min. at 1300 *g*) and sterilized by filtration as above. The volume of filtrate obtained was about 150 ml.

Preparation of freeze-dried enzymes. Preliminary experiments were carried out to determine the optimum concentrations of $(NH_4)_2SO_4$ for precipitation of the enzymes. All precipitations were carried out as near as possible to +1°. Freeze drying was carried out in 1 l. round-bottomed flasks at 0·1 mm. pressure using an acetone and solid carbon dioxide condenser. (1) *Cl. butyricum.* In a typical preparation an initial precipitation of the enzyme from 275 ml. of culture filtrate was carried out by addition of $(NH_4)_2SO_4$ solution (50% w/v; pH adjusted to 7·0 with NH_4OH) to 40% (w/v) and the precipitate freeze-dried. This precipitate (2·49 g.) was then dissolved in water (100 ml.; solution 1) and $(NH_4)_2SO_4$ solution added slowly to a concentration of 30% (w/v). After storing for 1·5 hr. the precipitate was compacted on the centrifuge in cold pots, washed with cold 30% (w/v) $(NH_4)_2SO_4$ solution, dissolved in 0·02 M-sodium citrate buffer (25 ml.; pH 7·0), and freeze-dried to give fraction *A*. Some $(NH_4)_2SO_4$ was added to the supernatant liquid to bring the concentration to 40% (w/v). The precipitate was removed after 4·5 hr., washed, dissolved in citrate buffer, as above, and freeze-dried to give fraction *B*. The initial activity of the solution (1) was 19 800 units, the activities of fractions *A* and *B* before freeze-drying were 17 000 and 1300 units respectively. After freeze-drying the weight of fraction *A* was 0·4609 g. and the activity 13 980 units. The weight of freeze-dried fraction *B* was 1·6296 g., but the activity was not determined. (2) *Streptococcus.* In a typical large-scale preparation $(NH_4)_2SO_4$ was slowly added to the filtrate (152 ml.) to a concentration of 40% (w/v). After standing at +1° for 14·5 hr. the fine precipitate was removed on the centrifuge, washed and dissolved in water (100 ml.). More $(NH_4)_2SO_4$ was then added to a concentration of 35% (w/v) and after storing at +1° for 4·5 hr. a fine suspension formed which was centrifuged off, washed with 35% $(NH_4)_2SO_4$, dissolved in 0·02 M-sodium citrate buffer (25 ml.; pH 7·0) and freeze-dried (fraction *A*). The weight of freeze-dried powder obtained was 0·6923 g. and the total activity 13 140 units compared with 13 200 units before freeze-drying. This was about half the activity of the original filtrate.

Fraction *A* from each filtrate was used in the following work. These powders kept their activity indefinitely when stored at +1°.

RESULTS

The action of the enzyme preparations on amylose and β-dextrin

Hobson *et al.* (1950) showed that by incubating equal amounts of α-amylase with (1) amylose and (2) β-dextrin and plotting the percentage change in *AV* (680 mμ.) of the amylose-iodine complex against that of the β-dextrin, a curve characteristic of the enzyme and independent of its concentration could be obtained. Two standard digests, both containing the same enzyme preparation, but one containing amylose and the other β-dextrin, were

incubated together, and, at equal periods after addition of the enzyme, portions were removed for determination of the *EV* (680 mμ.). The sample from the amylose digest was stained under the standard conditions given above, but that from the β-dextrin digest contained twice the normal concentrations of polysaccharide and was stained with twice the normal concentration of iodine to increase the accuracy of the measurements.

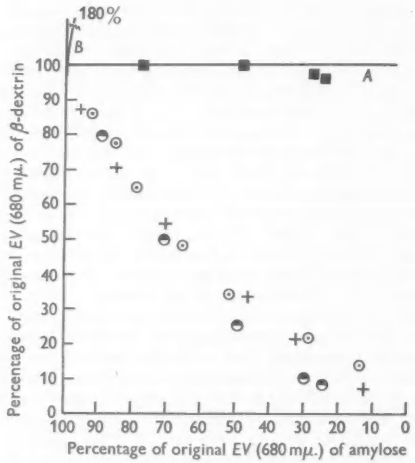

Fig. 1. The comparative action of the enzymes on amylose and β-dextrin. *A*, theoretical curve for β-amylase and Q-enzyme. *B*, curve for R-enzyme. Soya-bean β-amylase (■), clostridial amylase (+), salivary amylase (◓), at pH 7·0. Streptococcal amylase (⊙), at pH 6·0. All digests incubated at 35°.

Q-enzyme and β-amylase attack amylose but not β-dextrin so that a single straight line serves to represent the action of these enzymes. R-enzyme, which catalyses the hydrolysis of the α-1:6-linkages in β-dextrin and amylopectin, acts on β-dextrin with the formation of dextrins of higher blue value (Hobson, Whelan & Peat, 1951), and its action is represented by the curve shown in Fig. 1. α-Amylase such as salivary amylase is the only enzyme which attacks both substrates and the curve depicting its action is shown in Fig. 1 where the results of experiments using salivary α-amylase and the clostridial and streptococcal enzymes are plotted. Mixtures of α-amylase with other enzymes give curves of intermediate shape.

Optimum pH *and temperature of action of the enzymes.* The optimum pH of action was measured by determining the fall in *EV* (680 mμ.) of digests like the standard-activity digests but containing a sodium acetate-sodium veronal buffer the pH of which had been adjusted by the addition of 0·1 N-H_2SO_4 (5 ml. 0·143 M-sodium acetate-sodium veronal solution, *x* ml. acid, (18 − *x*) ml. water). The constituents were preheated to 35°, mixed, and the *EV* (680 mμ.) of portions of the digests were measured after incubation for 15 and 30 min. as in the determination of activity. The pH values of the digests were checked at the end of the incubation.

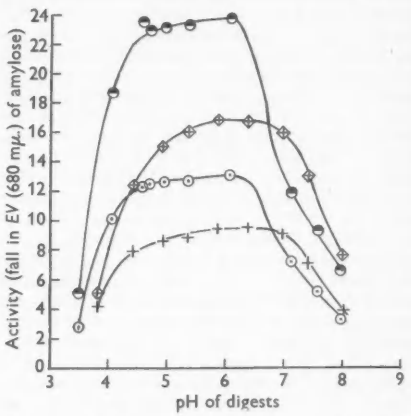

Fig. 2. The effect of pH on amylase activity at 35°. Incubation periods: clostridial amylase, 15 min. (⊙), 30 min. (◓); streptococcal amylase, 15 min. (+), 30 min. (⊕).

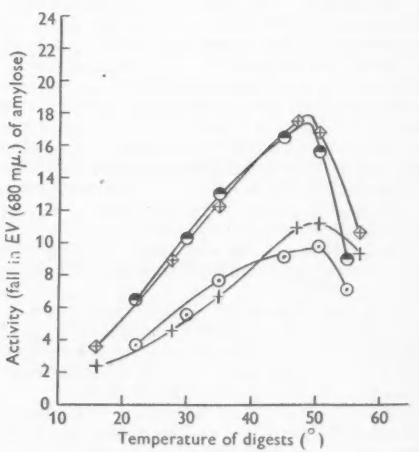

Fig. 3. The effect of temperature on amylase activity. Digests at pH 7·0. Incubation periods: clostridial amylase, 15 min. (⊙), 30 min. (◓); streptococcal amylase, 15 min. (+), 30 min. (⊕).

Both enzymes have a broad pH optimum. That of the streptococcal enzyme under these conditions lies between 5·5 and 6·5 and that of the clostridial enzyme between 4·6 and 6·1 (Fig. 2). This latter value is similar to the optimum pH range of

crystalline *B. subtilis* enzyme (5·3–6·8; Meyer *et al.* 1947), partially purified *B. subtilis* amylase (5–6·5; Di Carlo & Redfern, 1947*a*) and *B. polymyxa* amylase (6·2–7·5; Rose, 1948).

The optimum temperature was determined by incubating the standard activity digests at different temperatures and determining the fall in *EV* (680 mμ.) after 15 and 30 min. in the usual manner.

The results in Fig. 3 show that both enzymes have the same optimum temperature of 48 ± 1° under the digest conditions employed. For subsequent experiments a temperature of action of 35° was chosen for both enzymes.

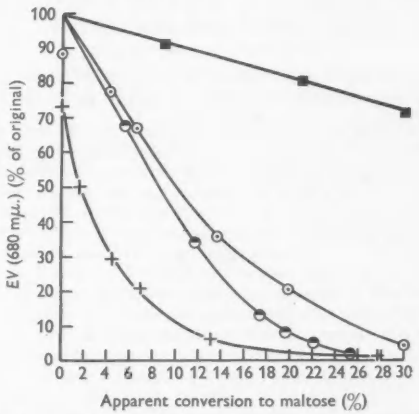

Fig. 4. The relationship between *EV* (680 mμ.) and percentage apparent conversion to maltose at 35° for amylose degraded by the clostridial amylase at digest pH values of 4·8 and 7·0 (+); streptococcal amylase at pH 6·0 (⊙); salivary amylase at pH 7·0 (◓). Typical β-amylase curve (■).

The action of the enzymes on amylose

The relationship between absorption value and percentage apparent conversion to maltose. An amylose of blue value 1·21 was used in these experiments to facilitate comparison of the results with those obtained by Whelan & Nasr (1951), as it was found that slightly different curves were obtained when amylose fractions of different blue values were used. Digests of the standard proportions, but with the total volume increased to 42 ml., were employed. A buffer of pH 7·0 was used for salivary α-amylase and two, of pH 7·0 and 4·8, for the clostridial amylase, the same curve being obtained at both pH values. For the streptococcal enzyme a buffer of pH 6·0 was used. Withdrawals for measurement of *EV* (680 mμ.) (see Methods section), and for measurement of reducing power, as maltose, were made alternately at noted intervals and from the progress curve of fall in *EV* with time the values corresponding to the respective reducing powers were found.

The results are shown in Fig. 4, a typical curve obtained with β-amylase being included for comparison.

Tests for the presence in the preparations of enzymes other than α-amylase

β-Amylase, Q- and R-enzymes. The results obtained from the comparison of the action of the enzyme preparations on amylose and β-dextrin obviate the presence of more than traces of β-amylase, Q- and R-enzymes.

Maltase. The polysaccharide solution in the standard digests was replaced by a maltose solution of the same concentration. The action of the clostridial enzyme on maltose was tested at pH values of 4·8 and 7·0 and that of the streptococcal enzyme at pH 6·0. Portions of the digests were removed for determination of the reducing powers, as maltose, at approximately 10 hr. intervals up to 48 hr.

No change in reducing power was found and the enzyme preparations were therefore free from maltase. Quantitative determinations, using portions of the digests as enzyme source, showed that the enzymes in the digests containing maltose retained their initial activity after incubation for 48 hr.

Phosphorylase. Phosphorylase activity was determined using the method of Green & Stumpf (1942) except that since the phosphorylase activity of the enzyme preparations was small, incubation was carried on for 1 hr. before the deactivation of the digests by addition of trichloroacetic acid. For determination of the initial concentration of inorganic phosphate a digest containing all the constituents but the enzyme was incubated in parallel, then trichloroacetic acid was added, and, immediately after, the enzyme preparation. Both digests were centrifuged and the inorganic phosphate in a suitable sample determined by the method of Allen (1940).

Experiments using the enzyme fractions obtained at 30 and 40% (w/v) concentrations of ammonium sulphate in the preliminary fractionation of the clostridial amylase showed that the fraction precipitated by 30% w/v $(NH_4)_2SO_4$ had a very slight phosphorylase activity, but that the fraction precipitated by 40% w/v $(NH_4)_2SO_4$ had three times as much. For the determination of the phosphorylase activity of the freeze-dried preparations the enzyme was used in a concentration of 220 α-amylase units/digest. The phosphorylase activity of the streptococcal amylase preparation was of the order of 1 unit/13 000 α-amylase units, and of the clostridial amylase 1 unit/10 000 α-amylase units.

Phosphatase. The composition of digests and the experimental conditions were the same as for the determination of phosphorylase activity except that 0·1M-sodium α-glycerophosphate replaced 0·1M-glucose-1-phosphate. Neither enzyme preparation liberated inorganic phosphate from the glycerophosphate. α-Glycerophosphatase activity is therefore absent under these conditions.

Invertase. The polysaccharide in the standard digests was replaced by a sucrose solution of the

same concentration. The buffers used were of pH values 4·8 and 7·0 for the clostridial amylase and 6·0 for the streptococcal enzyme. After incubation for 20 hr. none of the digests showed any reducing power thereby indicating the absence of invertase.

The effect of chloride and calcium ions on the activity of the enzymes

Chloride ions. Two digests were prepared as for the activity determinations except that one contained sufficient NaCl to bring the final concentration to 0·14 M. The digests were incubated at 35° for 30 min. when portions (1 ml.) were removed for determination of *EV* (680 mμ.) as in the determination of activity.

Calcium ions. Two digests were prepared each containing amylose solution (7 mg.; 3·75 ml.), 0·2 M-sodium acetate solution the pH of which was adjusted to 7·0 (1·5 ml.) and enzyme solution (0·75 ml.). In addition, one digest contained $CaCl_2$ solution (1 ml.) to give a final concentration of 0·07 M, and the other NaCl solution (1 ml.) such that the final concentration was 0·14 M. The digests were incubated at 35° and at 30 min. the *EV* (680 mμ.) of portions (1 ml.) of each were measured.

The results are shown in Table 1. The presence of calcium ions causes the slightly higher *EV* of the samples from digests containing calcium chloride. A similar effect is found with starch-iodine solutions containing calcium nitrate. Neither enzyme is activated by calcium or chloride ions under the conditions employed, but in some (unreported) experiments it was found that dilute solutions of the enzymes were stabilized by the presence of small concentrations of calcium ions.

The hydrolysis of starch and starch fractions by the enzymes

Solutions of the amylose, and amylopectin from potato starch were prepared and incorporated in digests of the standard proportions containing buffers of pH values 4·8 and 6·0 for the clostridial amylase and 6·0 for the streptococcal amylase. The digests were incubated at 35°, and, at intervals, the reducing sugars present were determined and expressed as maltose. The activities of the enzymes in the digests were determined at intervals.

After 27 hr. the streptococcal enzyme retained its initial activity in the digests (4·7 units/1 ml.), but at 72 hr. the activity had fallen to 2·9 units/1 ml., and at 118 hr. it was 0·8 unit/1 ml. The clostridial amylase retained its full activity at 24 hr., and the activity at 118 hr. was 1 unit/1 ml. At 72 hr. fresh enzyme was added to portions of each digest containing amylose and, to the digests containing the clostridial amylase, starch and amylopectin to bring the activity to the initial value; the extents of conversion to reducing sugars did not, however, markedly increase above those of the original digests. The apparent conversions to maltose of the different substrates by the two enzyme preparations are shown in Figs. 5 and 6; in each case the values found for starch are almost exactly those calculated from the extent of the hydrolysis of the two constituents, amylose and amylopectin.

Identification of the main products from the hydrolysis of amylose by the streptococcal-enzyme preparation

A standard digest containing 28 mg. of amylose was incubated at 35°, the reducing power being determined at 21 and 23·5 hr. (90·7% apparent maltose). At 27 hr. 20 ml. of the digest were shaken alternately with cation- and anion-exchange resins (Amberlite 1 R 100/8 and 1 R 4 B; Rohm and Hass Co., Philadelphia, U.S.A.) to remove the buffer and the solution evaporated to dryness under reduced pressure at 45°. The resulting residue was dissolved in water (0·5 ml.) and spots of this solution, to the total volumes given below, were layered on to the starting line of a strip of Whatman no. 1 filter paper. The first chromatogram had spots consisting of approximately 0·01 ml. of amylolysis products, and 0·01 ml. of amylolysis products mixed with 0·005 ml. of 1% glucose solution, together with reference spots of 0·002 ml. of 1% glucose, 0·002 ml. of 1% maltose and 0·002 ml. of 1% maltotriose. The chromatogram was developed with a solvent consisting of A.R. amyl alcohol (British Drug Houses Ltd.), pyridine and water in the proportions by volume of 7 : 7 : 6 (Jeanes, Wise & Dimler, 1951) for 20·75 hr. at 20°; the solvent, which had run 32 cm., was then dried off, and the chromatogram replaced and developed for a further 19·5 hr., the solvent running the same distance. After drying, the paper was coloured by spraying with a benzidine reagent (Bacon & Edelman, 1951).

Three spots corresponding to glucose, maltose and maltotriose were found in the α-amylolysis products. A similar chromatogram was developed with the top layer of a benzene, *n*-butanol, pyridine, water mixture (in the proportions by volume 10:50:30:30)

Table 1. *The effect of calcium and chloride ions on the activity of the enzymes*

(Digests 1 contained amylose (7 mg.), 0·2 M-sodium citrate (1·5 ml., pH 7·0), and enzyme in a total volume of 7 ml. In digests 2 the sodium citrate was replaced by sodium acetate, and the enzyme activity differed from digests 1. The *EV* gives a measure of the enzyme activity; see text. Initial *EV* of amylose, 42·0.)

	EV (680 mμ.) of amylose after incubation for 30 min. at 35°			
	1		2	
Salts added to digests	0·14 M-NaCl	No salts	0·14 M-NaCl	0·07 M-$CaCl_2$
Source of the enzyme				
A rumen *Streptococcus*	35·0	35·0	32·6	34·1
Clostridium butyricum	34·5	34·5	33·8	35·2

for 44·5 hr. at 20°, the solvent being allowed to drip off the bottom of the paper, and after drying was coloured with the benzidine reagent. Again spots corresponding to glucose, maltose and maltotriose were found in the α-amylolysis products, the separation of the sugars being greater in this solvent. No material of chain length greater than maltotriose was detectable on these chromatograms. The most abundant products of the hydrolysis are thus glucose, maltose and maltotriose, the intensity of the spots suggesting that the maltotriose was present in large amount, and the glucose in comparatively small amount.

The action of the enzyme preparations on maltotriose and maltotetraose

The maltotriose and maltotetraose used were isolated from the end products of hydrolysis of amylose by the clostridial amylase by chromatography on charcoal in the manner of Whistler & Durso (1950) and Bailey, Whelan & Peat (1950). The maltotriose had $[\alpha]_D + 168°$ (in water) and an apparent chain length (from the copper-reducing power measured as maltose) of 2·82 glucose units and gave the theoretical amount of glucose on acid hydrolysis. The maltotetraose had $[\alpha]_D + 176°$ and an apparent chain length of 4·2 and was hydrolysed to the theoretical amount of glucose. No carbohydrate impurities could be detected by chromatography and a graph of $\log (1/R_F - 1)$ per chain length of these sugars with glucose and maltose gave a straight line. The digests were of the same proportions as the standard digests, but containing a known amount (approx. 14 mg.) of the sugar. A buffer of pH 4·8 was used for the clostridial enzyme and of pH 6·0 for the streptococcal enzyme. The reducing power of digests containing maltotriose was measured up to 22 hr. in the case of the clostridial enzyme, and a further reading was taken at 51 hr. in the case of the streptococcal amylase. No increase in reducing power occurred. The results for the digests containing maltotetraose are shown in Table 2. The maltotetraose is slowly hydrolysed. The standard tests showed that the enzyme in all the digests was active after the last determinations of reducing power had been made.

Table 2. *The hydrolysis of maltotetraose by the bacterial amylases*

(The digests were incubated at 35° at pH values of 4·8 for the clostridial enzyme and 6·0 for the streptococcal enzyme, and contained maltotetraose (14 mg.), 0·2 M-sodium acetate (3 ml.), enzyme (66 units), water to 14 ml.)

Source of enzyme	Time of incubation	Apparent conversion of tetraose to maltose (%)
Clostridium butyricum	2 min.	37·1
	4·25 hr.	59·3
	20 hr.	81·5
A rumen *Streptococcus*	2 min.	46·5
	21 hr.	80·3
	45·5 hr.	91·9
	94·5 hr.	95·6

DISCUSSION

The simultaneous action on amylose and β-dextrin of the two enzymes described in this paper (Fig. 1), together with the results obtained by a comparison of the change in iodine stain and the apparent conversion to maltose of amylose, indicate that the enzymes are of the α-amylase type. With both enzymes the iodine stain of the polysaccharide changes from blue, through purple and red and then disappears during the hydrolysis of amylose or starch, a result which is given by α- and not by β-amylases. The two enzymes differ in the extent of conversion of amylose to sugars, during the initial stages of the hydrolysis. The enzyme of the rumen strain of *Cl. butyricum* shows a similarity to that of the enzyme from the pig caecum strain examined by Whelan & Nasr (1951), but differs from the streptococcal enzyme, which is similar to that of salivary α-amylase. This difference between the enzymes could be explained by assuming that the streptococcal and salivary α-amylases make a more endwise attack on the amylose chain splitting off low molecular weight sugars with a high reducing power and leaving dextrins of long chain length and high blue value. The clostridial enzymes, on the other hand, rapidly degrade amylose in a more random manner forming dextrins of short chain length and low blue value. Thus at any given apparent conversion to maltose the clostridial amylase digests have a lower *EV* (680 mμ.) than the salivary and streptococcal amylase digests. Further work to identify some of these intermediate products is in progress. This difference between the enzymes is maintained up to the end of the hydrolysis, the clostridial enzymes giving lower conversion limits than the salivary and streptococcal enzymes. On comparison of the extents of conversion of amylose to reducing sugars there is also, however, a difference between the amylases of the two strains of *Cl. butyricum*. The amylase from the pig caecum strain of organism hydrolysed starch and amylose to 65–66% apparent maltose, a figure which was not increased on prolonged incubation or addition of more enzyme. The amylase from the sheep rumen strain of *Cl. butyricum* rapidly hydrolyses amylose to about 80% apparent maltose and this extent of conversion slowly increases on prolonged incubation (Fig. 5). Starch and amylopectin are hydrolysed to smaller extents. The streptococcal

enzyme, however, again appears to be similar to salivary α-amylase in the final apparent conversion to maltose. Roberts & Whelan (1951) have recently reported a final conversion of amylose to 90% apparent maltose by salivary α-amylase. With the streptococcal enzyme also starch and amylopectin have lower extents of conversion to sugars than amylose. This is most likely due to the presence of α-1:6-linkages in amylopectin which are not hydrolysed by the α-amylases and which act as obstructions to the enzymes and lead to the formation of dextrins of molecular weight greater than maltose and maltotriose; these dextrins are then slowly hydrolysed. This is in accordance with the results of other workers (Meyer & Gonon, 1951; Myrbäck, 1950) who concluded that α-amylases do not hydrolyse α-1:6-linkages.

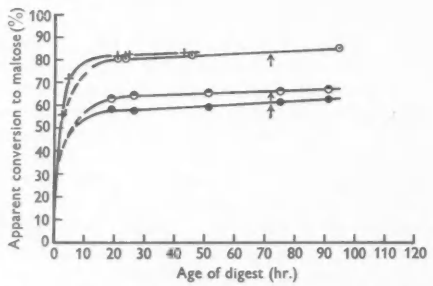

Fig. 5. The hydrolysis of starch fractions by the clostridial amylase at 35°. Digests at pH 4·8; amylose (⊙), starch (◓), amylopectin (●). Digest at pH 6·0; Amylose (+). Addition of enzyme to bring the activity to the initial value (↑).

In a later publication it is hoped to describe the isolation of the end products of the hydrolysis of amylose and starch by these enzymes, and a preliminary fractionation has shown that the clostridial enzyme produces from amylose a mixture of sugars of which maltose and maltotriose are the main constituents, together with a little glucose and maltotetraose. The end products of the action of the streptococcal enzyme on amylose were shown by paper chromatography to contain glucose, maltose and maltotriose. As mentioned above, neither enzyme hydrolyses maltose or maltotriose, so the slow increase in reducing power on prolonged incubation of the digests containing starch and starch fractions is most likely due to the degradation of remaining traces of maltotetraose, which is hydrolysed by the enzymes (Table 2), and other dextrins of slightly longer chain length. Whelan & Nasr (1951) reported that a preliminary investigation of the products of hydrolysis of amylose by the pig caecum clostridial amylase showed the presence of maltose and maltotriose, and later Roberts & Whelan (1951) isolated the end products of hydrolysis of amylose by salivary α-amylase and showed these, again, to be maltose and maltotriose. It would thus appear that maltotriose is a main product of the degradation of amylose by all these amylases. The difference in the final reducing powers of the digests containing amylose would then be mainly due to differences in the proportions of maltose, maltotriose and glucose present. In the products of hydrolysis of amylose these enzymes differ from the malt α-amylase and pig pancreatic amylase studied by Meyer & Gonon (1951) which are stated to give maltose and glucose as the end products of hydrolysis of amylose, maltotriose being a comparatively slowly hydrolysed intermediate product.

In further experiments it was found that the two organisms grew on media containing glucose,

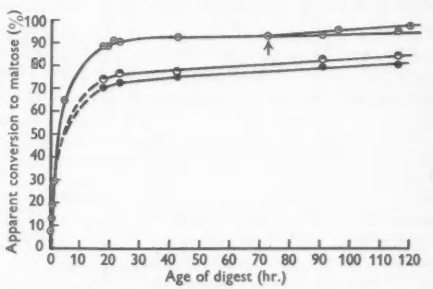

Fig. 6. The hydrolysis of starch fractions by the streptococcal amylase at 35°. Digests at pH 6·0; amylose (⊙), starch (◓), amylopectin (●). Addition of enzyme to bring the activity to the initial value (↑).

maltose or maltotriose as the only carbohydrates and that acids were produced by fermentation of these sugars. Thus these organisms can use as energy sources the main products of the hydrolysis of starch by their isolated extracellular amylases.

SUMMARY

1. Starch-degrading enzymes have been isolated in the form of stable freeze-dried powders from cell-free filtrates of cultures of sheep rumen strains of *Clostridium butyricum* and a *Streptococcus* grown in media containing dissolved starch. Their properties have been examined.

2. The enzymes belong to the group of α-amylases. The hydrolysis of starch and starch fractions by the enzymes has been investigated and the two enzymes have been shown to differ in the extents of conversion of these substrates to reducing sugars. The branch linkages in amylopectin act as obstructions to both enzymes.

3. The presence in the enzyme preparations of β-amylase, maltase, invertase, phosphatase or Q-

and R-enzymes could not be detected, but small traces of phosphorylase were present.

4. Maltose and maltotriose with some glucose are the main end products of the hydrolysis of amylose by these enzymes. Neither amylase hydrolyses these sugars, but maltotetraose is degraded by both enzymes.

5. A simple method for determination of α-amylase activity is described.

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The Free Amino-acids Occurring in the Body Tissues and Blood of the Rat and of the Cow

By D. M. WALKER
*University College, London**

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Previous to the work reported here, few papers have been published showing the distribution of free amino-acids among the tissues of any one animal. Awapara, Landau & Fuerst (1950) have reported on the free amino-acids in rat tissues, while Gordon (1949) has investigated the intracellular fluid of calf embryo muscle and compared its free amino-acid composition with that of adult ox muscle and adult ox serum.

* Present address: Rowett Research Institute, Bucksburn, Aberdeenshire.

EXPERIMENTAL

Total N. Micro-Kjeldahl estimations were carried out on the fresh tissue.

Preparation of protein-free filtrates. These were prepared from rat and cow tissues, blood and urine. The rats used in the experiments were of no specified age, but were mature males of approx. 400 g. body wt. They were slaughtered by dislocation of the neck and the organs removed as quickly as possible. Cow tissues were obtained from the slaughterhouse and were removed from the body as soon as possible after the death of the animal. They were then packed in ice for transport to the laboratory where they were immediately

minced and extracted. The whole process was always completed within 2 hr. of slaughter. For the preparation of protein-free filtrates the methods of Awapara (1948) and of Roberts & Frankel (1949) were used for cow and rat tissues respectively. It was necessary with such tissues as muscle, heart and lung in the cow to pass them through an ice-cold mincer before homogenization in the Waring Blendor.

An examination of both blood cells and plasma was carried out in the following manner. Fresh blood was withdrawn from the dorsal aorta of the rat or the jugular vein of the cow, heparin being present in the receiving vessel to prevent clotting. The blood was centrifuged as soon as possible after collection. The plasma and cells were pipetted off separately and the cells haemolysed with an equal volume of distilled water. Proteins in the plasma and haemolysed cells were precipitated in several different ways. Ethanol precipitation was effected by treating 1 ml. of plasma with 10 ml. of 95% ethanol, allowing to stand for 15 min. and then centrifuging down the precipitate. The ethanol was removed from the supernatant by evaporation in a current of warm air. The residue was taken up in a known volume of water, desalted in the apparatus of Consden, Gordon & Martin (1947) and quantities equivalent to 250 μl. of blood cells and plasma were applied to chromatograms. Ultrafiltration and tungstic acid precipitation (Hier & Bergeim, 1945) were also tried. On comparing the results obtained by these three methods the ethanol precipitation was found to produce the cleanest chromatograms. However, it appeared that with this method of precipitation losses of amino-acids occurred and glutathione especially was in smaller concentration when compared with ultrafiltration.

Urine was collected from cows in milk and young calves still receiving an all-milk diet. A few samples were also obtained from heifers of 6 and 12 months of age. Provided a small enough quantity (about 15 μl.) was placed on the chromatogram, no interference from salts was encountered.

Chromatographic techniques. Methods of chromatography were similar to those described by Dent (1948). One-dimensional chromatograms were run in butanol-acetic acid on Whatman no. 4 paper. Markers of histidine, tyrosine and arginine were run side by side with various concentrations of the extract. The paper was then dried and the presence of histidine, tyrosine and arginine established by means of specific colour reactions.

Whatman no. 1 papers (20 × 20 cm.) were used for small chromatograms with the frame of Datta, Dent & Harris (1950). Two-dimensional runs in phenol and the collidine mixture (collidine/lutidine/water: 1/1/1) were carried out, with NH_3 in the phenol tank and diethylamine in the collidine tank. Various concentrations of the extract equivalent to 0·5, 1·0 and 1·5 mg. N of the fresh tissue were applied to chromatograms. The squares were then sprayed with 0·1% ninhydrin in *n*-butanol and heated at 100° for 1 min.

Large two-dimensional chromatograms were run on Whatman no. 4 (18 × 22 in.) paper in phenol and collidine mixture, the phenol developing along the 18 in. edge and the collidine/lutidine following at right angles in the longer direction. A quantity of extract, equivalent to 5 mg. N in fresh tissue was applied.

Hydrolysis of extracts. The extract was hydrolysed with 6 N-HCl for 15 hr. at 100° in a closed glass phial. The hydrolysate was taken to dryness in a current of hot air, taken up in a known volume of water and desalted. Quantities of extract equivalent to 0·5, 1·0 and 1·5 mg. N in the fresh tissue were then run on small chromatograms.

Oxidation of extracts. The extract was oxidized according to the method of Dent (1948). Quantities of extract equivalent to 0·5, 1·0 and 1·5 mg. N of the fresh tissue were then run on small chromatograms.

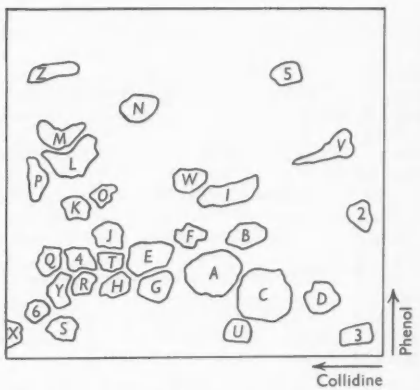

Fig. 1. 'Map of spots' showing those substances reacting with ninhydrin and occurring in the various biological extracts.

A	Glycine	*Q*	Proline
B	Serine	*R*	γ-Aminobutyric acid
C	Glutamic acid	*S*	Lysine
D	Aspartic acid	*T*	Histidine
E	α-Alanine	*U*	Ethanolaminephosphoric acid
F	Threonine	*V*	Cystine
G	Glutamine	*W*	Unknown—above left of taurine
H	β-Alanine and glyceryl-phosphorylethanolamine	*X*	'Fast-phenol'
I	Taurine	*Y*	Carnosine
J	α-Aminobutyric acid	*Z*	'Cow-spot'
K	Valine	2	Cysteic acid
L	Leucines	3	Glutathione
M	Phenylalanine	4	Unknown—left of α-alanine
N	Tyrosine	5	Chloride
O	Methionine sulphone	6	Arginine
P	Ethanolamine		

RESULTS

Table 1 shows those amino-acids which were found in the separate tissues of the rat and the cow after the various treatments, while Fig. 1 gives a map of the amino-acids and other ninhydrin-reacting substances.

An examination of the results obtained with the rat tissues reveals that the following ninhydrin-reacting substances occur in the free state in all tissues: glycine, glutamic acid, aspartic acid, α-alanine, glutathione, glutamine, taurine, leucines, serine, lysine, valine, proline, threonine, tyrosine,

Table 1. *Free amino-acids in an ethanol extract of rat and cow tissues*

(* = after hydrolysis of ethanol extract; † = after oxidation of ethanol extract; + + +, dense; + +, medium; +, small; ?, doubtful; −, none.)

	Brain	Lung	Heart	Liver	Muscle	Kidney	Spleen	Blood cells	Blood plasma
					Rat				
Glycine	+ + +	+ + +	+ +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
α-Alanine	+ +	+ +	+ +	+ + +	+ + +	+ +	+ +	+ +	+ +
Serine	+ +	+	+	+	+ +	+ +	+ +	+ +	+ +
Threonine	+	+	+*	+	+	+	+	+	+ +
Valine	+	+	+	+	+	+ +	+ +	+ +	+ +
Leucines	+	+	+	+	+	+ +	+ +	+ +	+ +
Phenylalanine	+	+	+*	+	+	+	+	+	+ +
Tyrosine	?	+ +	?	+	+ +	+	+ +	+	+
Proline	+*	+	+	+	+	+	+	+	+
Aspartic acid	+	+ +	+ +	+ +	+	+ +	+ +	+ +	+ +
Glutamic acid	+ + +	+ +	+ + +	+ +	+ +	+ + +	+ + +	+ +	+ +
Histidine	+	+	+	+ +	+ +	+	?	+	−
Arginine	−	−	−	−	−	?*	−	+	+
Lysine	+	+	+	+	+	+	+	+ +	+ +
β-Alanine	+*	+*	+*	?*	+ + +*	+ +*	+*	−	+*
α-Aminobutyric acid	−	−	−	+	−	−	−	+	+
γ-Aminobutyric acid	+ +	+	+	+*	−	?*	?*	+	+
Taurine	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+	+
Methionine sulphone	−	+†	+ +†	−	−	+†	+†	+†	+†
Cysteic acid	+†	+†	?†	+†	−	+†	+†	+†	−
Glutamine	+ + +	+ +	+ + +	+ + +	+ + +	+ +	+ +	+	+
Gluthathione	+ + +	+ +	+	+ + +	+ +	+ +	+ +	+ +	−
Histamine	−	−	−	−	−	−	−	−	−
1-(3)-Methylhistidine	−	−	−	−	+ +*	−	−	−	−
Carnosine	−	−	−	−	+ + +	−	−	−	−
Ethanolamine	−	−	−	+*	−	−	−	?*	−
Ethanolaminephosphoric acid	+ +	+ +	+	+ +	?	+ + +	+ + +	−	−
Glycerylphosphorylethanolamine	−	−	−	+ +	−	−	−	−	−
Unknown—left of α-alanine	−	−	−	−	−	−	−	−	−
Unknown—above left of taurine	−	−	−	−	−	−	−	−	−
					Cow				
Glycine	+ +	+ + +	+ +	+ + +	+ +	+ + +	+ + +	+ + +	+ + +
α-Alanine	+ +	+ +	+ + +	+ + +	+ + +	+ +	+ +	+ +	+ +
Serine	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
Threonine	+†	−	−	−	−	+	+†	+	+
Valine	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
Leucines	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ + +	+ +
Phenylalanine	+	+	+ +	−	−	+	+	+	+
Tyrosine	+ +	+ +	+ +	+	−	+ +	+	+	+
Proline	+	+ +*	+	+	+*	+ +	+	+ +	+
Aspartic acid	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+	+
Glutamic acid	+ + +	+ +	+ + +	+ + +	+ +	+ + +	+ + +	+ +	+ +
Histidine	+ +	+	+ +	+ +	+ +	+ +	+	+ +	+ +
Arginine	−	−	−	−	−	+	?†	−	+
Lysine	+	+ +	+ +	+	+ +	+ +	+	+ +	+
β-Alanine	+*	+*	+ +*	+ +*	+ + +*	+ +*	+*	+ +*	+*
α-Aminobutyric acid	+	+	−	+ +	+*	+	+	+	+ +
γ-Aminobutyric acid	+ +	+	+*	+*	−	+*	+	+	+
Taurine	+ +	+ + +	+ +	+ +	+ +	+ + +	+ + +	+	+ +
Methionine sulphone	+†	−	+ +†	+†	+†	+ +†	+ +†	+ +†	+ +†
Cysteic acid	+ +†	−	−	+ +†	−	+ +†	+ +†	+ +†	−
Glutamine	+ + +	+	+ + +	+ +	+ + +	+	+ +	+	+ +
Glutathione	+ +	+ +	+ +	+ + +	+ +	+ +	+ +	+ + +	−
Histamine	−	−	+	+	−	−	−	−	−
1-(3)-Methylhistidine	−	−	−	−	+ +*	−	−	−	−
Carnosine	−	−	−	−	+ + +	−	−	−	−
Ethanolamine	+*	−	−	+ +*	−	+*	+*	−	−
Ethanolaminephosphoric acid	+ +	+ +	+ +	+ +	+	+ +	+ +	+	−
Glycerylphosphorylethanolamine	+	−	−	+ + +	−	+ +	+	−	−
Unknown—left of α-alanine	−	−	−	−	+	−	−	−	−
Unknown—above left of taurine	+	−	+*	+†	+	+	+	+ +*	+ +*

histidine, phenylalanine and ethanolaminephosphoric acid.

The cow tissues show several unknown spots and in muscle a ninhydrin-positive substance appears to the left of α-alanine. This may be identical with the spot which Gordon (1949) has reported as methionine sulphoxide. However, it withstood oxidation with H_2O_2 in the presence of ammonium molybdate, while Dent (1948) has stated that methionine sulphoxide will be converted into the sulphone under these conditions. After hydrolysis of a muscle extract a green spot in the position of methylhistidine was present. This would suggest that the peptide anserine was present in the ethanol extract, though owing to the large amount of carnosine present this would not be seen. Zapp & Wilson (1938) have demonstrated the presence of both anserine and carnosine in ox muscle and have shown that there is almost twice as much carnosine as anserine in most muscles.

In cow liver the unknown spot (Fig. 1, spot *H*) appears to be the same as that demonstrated by Campbell (1951) in pig liver. On hydrolysis of the latter, an ethanolamine spot appears, and the unknown has been identified as glycerylphosphorylethanolamine (Campbell & Work, 1952). This material was also seen in small amounts in brain, kidney and spleen. Brain tissue showed a large amount of γ-aminobutyric acid. Although this amino-acid also occurs in other tissues it does not do so in such relatively large quantities. In the spleen tissue examined there appeared to be some relationship between threonine and the unknown spot situated in a position above-left of taurine. Sometimes on the small chromatograms a threonine spot was visible, yet when the same material was run on a large chromatogram the unknown spot was seen and no threonine spot. It is not yet known whether it is threonine varying its position in the collidine mixture or whether the threonine is changed during running to a different form. The unknown spot was seen on both hydrolysed and unhydrolysed extracts and has been noted on both large and small chromatograms.

A small sharply defined spot was often seen on the bottom left-hand corner of the chromatogram, (Fig. 1, spot *X*) on the oxidized and ethanol extract squares. It may be referred to as 'fast-phenol' as it moves very little in the collidine mixture. It has been found in most tissues and in blood, but has not been seen after hydrolysis. It may be identical with the 'nephrosis-peptide' mentioned by Dent (1948) but has not been further studied. It has been noted in spleen, brain, liver, kidney, heart, blood cells and plasma but not in lung and muscle extracts. There is also a commonly occurring purple spot in position 5 (Fig. 1). This is due to the presence of chloride in the extract, which reacts with the diethylamine in the collidine tank and then gives a positive ninhydrin reaction.

The Pauly reaction with lung tissue extract showed a strong spot for tyrosine with only a weak spot for histidine. This was in direct opposition to the general finding for all other tissues tested and for blood cells and plasma, where histidine was present in greater amount. Table 2 has been constructed giving numerical values to the depth of colour obtained with equivalent amounts of different tissues, as judged by eye. A further spot given by the Pauly reaction in heart and liver was probably due to histamine.

Table 2. *Concentration of histidine and tyrosine in blood and tissues of the cow*

(Values comparative, as judged by eye.)

	Histidine	Tyrosine
Muscle	10	—
Spleen	1	1
Heart	5	2
Brain	6	1
Liver	2	2
Kidney	2	2
Lung	1	6
Blood cells	6	1
Blood plasma	1	1

The main difference noted between blood cells and plasma was the absence of glutathione in the latter. Ethanolaminephosphoric acid was present in traces in the cells but was not seen in plasma. Different samples of blood showed variation in the proportions of different amino-acids. Taurine was sometimes present only in traces in the cells and plasma, while at other times there were high concentrations. Aspartic acid always seemed to be present in small amount.

Undesalted cow urine gave two ninhydrin-reacting spots with 20 μl. of urine. Glycine appeared as a weak spot with an unknown spot, to be termed 'cow-spot', in a position on the chromatogram above phenylalanine and giving a purple colour (Fig. 1, spot *Z*). On desalting the urine this unknown spot disappeared and the glycine spot appeared larger in size and deeper in colour. Attempts were made to concentrate the spot by cutting it out from large sheets, but the material was extremely labile and often disappeared during the process. When calf urines were examined, the 'cow-spot' was not present but the urine contained the following amino-acids: glycine, glutamic acid, taurine, α-alanine and glutamine. Aspartic acid was found in some calf urines. The urine of 6- and 12-month-old heifers contained the 'cow-spot' so that it is probably of dietary origin, its absence from the calf urine being due to the all-milk diet. The 'cow-spot' was present in all cow urines analysed, but owing to its lability it was not studied from the point

of view of breed of animal, time of year when it appeared, effect of diet, etc. When the urine was stored for some time after collection the 'cow-spot' again disappeared and the glycine spot only was present.

DISCUSSION

Although many individual tissues have been examined for their free amino-acids only two papers have come to the notice of the author where several different tissues of any one animal have been studied. The paper by Awapara *et al.* (1950) on the free amino-acids of rat tissues includes many results which are at variance with the work reported here. It is unnecessary to discuss all the differences, but it may perhaps be relevant to mention the most widely divergent results. Awapara *et al.* (1950) detected no serine, valine, lysine, histidine or carnosine in muscle. They did, however, find methionine and hydroxyproline. It is noticeable that they detected no leucines in any tissue, but found methionine in all of them. One may suppose that they have mistaken the leucine spot for methionine, for both amino-acids run to almost identical positions on a phenol-collidine chromatogram. The methionine can only be definitely identified by oxidizing it to its sulphone (Dent, 1948). Awapara *et al.* also state in their paper that the appearance of β-alanine and histidine upon acid hydrolysis of liver and skeletal-muscle extracts points to the existence of carnosine in these organs. It is possible that on the chromatograms of the unhydrolysed extract they have mistaken the carnosine for hydroxyproline since both give brownish yellow spots with ninhydrin, though the carnosine spot will go green in colour if heated to 105° (Dent, 1948).

The analyses of Gordon (1949) of ox muscle and ox serum agree quite satisfactorily with the results obtained in the present work. Arginine, tyrosine and phenylalanine were, however, not detected in the concentration of muscle extract applied to the chromatograms. Gordon also found tryptophan in the ox serum which he examined. This amino-acid was not recognized in the present work in any tissue. It is possible that loss of tryptophan occurred at some stage during the preparation of the extract.

SUMMARY

The free amino-acids occurring in various tissues and body fluids of the rat and cow have been detected using the technique of paper partition chromatography.

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The Fate of Intravenously Administered Coproporphyrin III in Normal and Lead-treated Rabbits

By M. WEATHERALL
Department of Pharmacology, London Hospital Medical College, London, E. 1

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Abnormally large amounts of coproporphyrin, mainly of type III, are usually found in the urine of mammals to which salts of lead have been administered. A three-carboxyl porphyrin has also been observed (Nicholas & Rimington, 1951; Weatherall & Comfort, 1952), and the latter authors have found evidence of a porphyrin containing five carboxyl groups. The quantity of coproporphyrin in the faeces is little or not at all increased, at least in man (Kark & Meiklejohn, 1941) and rabbits (Fischer & Duesberg, 1932; Weatherall, 1948 and unpublished observations; Schwartz & Zagaria, 1951), and the coproporphyrin which is present is predominantly of type I (Dobriner, 1937*a*; Vigliani & Libowitzky, 1937; Watson, 1937), as in normal subjects. The abnormal excretion of coproporphyrin is therefore

more or less confined to the urine. This is surprising, because injected coproporphyrin is normally mainly excreted in the bile (Dobriner, 1937*b*; Vigliani, Angeleri & Sano, 1938). Even in lead-poisoned patients only a small fraction of injected coproporphyrin can be accounted for in the urine (Vigliani, 1938). Earlier studies (Fischer & Hilmer, 1926; Grotepass & Hulst, 1935) suggesting that administered coproporphyrin is excreted by the kidneys lack sufficient detail to provide reliable evidence on the problem.

A possible explanation of these observations is that the coproporphyrin recovered from the urine is excreted not as such, but as a precursor, which changes to coproporphyrin after it has passed the kidney. Various observations, summarized by Watson, de Mello, Schwartz, Hawkinson & Bossenmaier (1951), show that at least part of the urinary coproporphyrin is excreted in this way, and that the precursor changes to the free porphyrin very easily during extraction. Watson *et al.* (1951) extracted rapidly fresh samples of urine from various sources and found up to two-thirds of the total coproporphyrin present as a precursor. Eriksen (1951) reported similar findings. Neither Watson nor Eriksen excluded the possibilities that conversion of the precursor could occur while the urine was accumulating in the bladder, or after collection and during their extractions. If the one-third of the coproporphyrin recovered which is supposedly 'preformed' had in fact developed during this time, it would have been in keeping with their observations.

If all the urinary coproporphyrin is excreted as a precursor, the discrepancy between the normal biliary excretion of injected coproporphyrin and the appearance of coproporphyrin in the urine in lead poisoning would be explained. The abnormal metabolite in animals exposed to lead would then be the precursor and not coproporphyrin itself. To examine this hypothesis, some observations have been made on the precursor in the urine of rabbits treated with lead. Also, coproporphyrin III has been administered to normal and to lead-treated rabbits and the amounts found in the urine and bile have been estimated.

METHODS

Administration of lead salts to rabbits. Male chinchilla rabbits weighing 1·7–2·5 kg. were used in all the experiments. They received an initial dose of $PbCl_2$ intravenously (0·05 m-mole/kg. given in 0·02 M solution at a rate of 2·0 ml./min.) and after a fortnight they were given a diet to which 250 parts of Pb, as $PbCl_2$, was added per million parts by weight of diet. This is roughly equivalent to a daily intake of 0·2 mg. Pb/kg. body weight. The duration of poisoning has been measured from the time of the priming dose. After some initial depression, the rabbits remained apparently well on this treatment, but excreted abnormally large amounts of coproporphyrin.

Cannulation of the bile duct. Rabbits were anaesthetized with pentobarbitone (Nembutal, Abbott) (25–40 mg./kg. intravenously initially, supplemented with smaller amounts or with ether by inhalation as required). The cystic duct was tied and the common bile duct was cannulated with a small glass cannula and the bile was collected in graduated tubes. Artificial respiration was given if necessary, though this was unusual. Blood pressure was recorded with a mercury manometer from the carotid artery. Bile flowed freely only when the blood pressure was above 60–70 mm. Hg, and nearly all the rabbits were given ephedrine hydrochloride (5 mg./kg. intravenously, repeated if necessary) and 0·9% (w/v) NaCl or 6·0% dextran in 0·9% NaCl (Dextran-Benger) to maintain their blood pressure above this level.

Extraction of coproporphyrin and its precursors. Coproporphyrin was extracted from urine and bile by the usual acetic acid-ether procedure, with one of two variants, called *A* and *B* below. In both procedures, except when otherwise stated, samples of 5–15 ml. of urine or bile were extracted twice with 15 ml. of ether. Glacial acetic acid (2–5 ml.) was added to the first portion of ether before extracting. The two ethereal extracts were combined and washed with 5 ml. 0·015 M-sodium acetate. In procedure *A* the ether was then extracted immediately with N-HCl until no more material fluorescing red or pink in ultraviolet light was obtained. In procedure *B*, the ether was washed once with 0·005% (w/v) aqueous I_2 before extraction with N-HCl (cf. Schwartz, Zieve & Watson, 1951). In each procedure the pH of the solution in HCl was raised to about 4, as judged by bromophenol blue as external indicator, with solid or a saturated solution of sodium acetate. The porphyrins were extracted with two or more 10 ml. portions of ether and from the ether with 0·07 N-HCl. Extracts were taken until they did not fluoresce red in ultraviolet light. In procedure *A*, all the portions of ether used in the extractions were kept on the bench for 18–24 hr. and again extracted with N-HCl. In this time red-fluorescent material commonly appeared, and occasionally still more was found after standing for another day. These extracts were neutralized and taken via ether to 0·07 N-HCl as before, and were estimated separately. The first portion included any coproporphyrin which had been extracted as such and an uncertain amount formed from precursor. The later portions contained only porphyrin formed from precursor after excretion. In some experiments, performed before the importance of the precursor was recognized, the ether was not kept and re-extracted and only the equivalent of the first portion was taken. This procedure is referred to as *A* −. When procedure *B* was used and the various portions of ether were kept as in procedure *A*, no additional red-fluorescent material developed on standing which could be extracted with HCl; it was therefore assumed that all the precursor in the sample was converted to free porphyrin by the wash with I_2. Extracts in 0·07 N-HCl prepared from bile were washed with 0·5 vol. of $CHCl_3$ twice, or more often if any trace of blue or green pigments was observed in the first wash before estimation. The porphyrins in 0·07 N-HCl were estimated in a Unicam spectrophotometer (λ, 401 mμ.) calibrated with a standard solution of coproporphyrin in the same solvent.

The extracts of 0·07 N-HCl were liable to contain porphyrins believed to have three and five carboxyl groups as well as coproporphyrin (Weatherall & Comfort, 1952). Their contribution to the total absorption at 401 mμ. has not yet been studied, and has been ignored as they are only a small fraction of the total porphyrin present. In order to detect

them, samples of the porphyrin after estimation were adsorbed on to acid-washed talc (Nicholas & Comfort, 1949), eluted with 0·5 N-NH_4OH in acetone and chromatographed on paper at 7° with 40% (v/v) water in lutidine (Nicholas & Rimington, 1949, 1951) in parallel with standard coproporphyrin or deuteroporphyrin.

Preparation of coproporphyrin. Urine from rabbits to which $PbCl_2$ had been administered was collected from galvanized-metal metabolism cages and was extracted as indicated above, except that larger volumes were used. The ether-soluble porphyrins so obtained were taken into ether and then to dryness. They were esterified with methanol saturated at 0° with dry HCl gas; dissolved in $CHCl_3$; washed repeatedly with 0·1 M-Na_2CO_3 and then with water; and chromatographed from benzene on Al_2O_3 (Nicholas, 1951). On developing the chromatograms with increasing concentrations of $CHCl_3$ in benzene, several fractions appeared. Details will be reported separately. The main porphyrin present was eluted with 30% (v/v) $CHCl_3$ in benzene, and was crystallized from $CHCl_3$ and light petroleum. The needle-shaped crystals appeared typical of coproporphyrin III and melted at 146°, remelting at 164° (uncorr.). A standard solution of coproporphyrin was prepared by leaving a weighed quantity (2 mg.) of the methyl ester in 7·5 N-HCl for 36 hr. at room temperature and then diluting with water to 0·6 N and storing in the refrigerator. The identity of the porphyrin was checked by its absorption spectra in ether and in HCl and by paper chromatography, when it ran as a single spot (R_F, about 0·65) in parallel with a sample of coproporphyrin kindly presented by Prof. C. Rimington and Miss R. E. H. Nicholas. For injection into rabbits the solution was nearly neutralized with 2·5 N-NaOH and made faintly alkaline with 0·5 M-$NaHCO_3$ immediately before use; it was diluted with water until it was approximately isotonic with 0·15 M-NaCl.

RESULTS

The presence of a precursor of coproporphyrin in urine

Experiments similar to those described by Watson *et al.* (1951) were performed on urine obtained immediately before extraction from lead-treated rabbits by suprapubic pressure. As exposure to ultraviolet light accelerates conversion of the precursor (Saillet, 1896; Watson *et al.* 1951), the experiments were done in a dark room lit by one 25 W. electric lamp about 1·7 m. from the work bench. Urine (5 or 10 ml.) was extracted once with 5–10 vol. of ether or ethyl acetate, so that foams did not form and separation occurred rapidly. The organic solvent was washed once with 0·015 M-sodium acetate and the free porphyrin was extracted with 4 × 2 ml. N-hydrochloric acid. Other experiments on comparable samples of urine showed that this removed all the initially detectable red-fluorescent material. The organic solvent was washed with 0·005% (w/v) iodine to convert any precursor remaining in it and was again extracted with N-hydrochloric acid. When the first set of extractions with N-hydrochloric acid was completed within 10 min. of the urine being obtained from the bladder, and the additional material obtained after washing the organic solvent with iodine was estimated separately, the mean amount of apparently preformed porphyrin was 51% (S.D. of nine observations on different samples of urine ± 19%) and the minimum 17%. When the extraction was performed more slowly, the amount of apparently preformed porphyrin was greater. The mean increment in samples extracted in 30 instead of 10 min. was 9% and in samples extracted in 90 min. was 21%. As expected, therefore, conversion of precursor was occurring in the conditions of these experiments and the estimates of preformed porphyrin are likely to be too large. Simple attempts to prevent the conversion were unsuccessful. It was impracticable to perform the extractions much more rapidly. The addition of reducing agents, such as ferrous sulphate, ascorbic acid or thioglycollic acid (all 0·01 M) to the urine had no substantial effect, nor did the use of ice-cold reagents. As the identity and exact behaviour of the precursor of coproporphyrin are not known, it is possible that the procedure here described did not extract all that was present. Also, some is likely to have been converted to coproporphyrin before the extraction of the latter was completed. Both sources of error tend to overestimate the proportion of preformed porphyrin. The present findings therefore confirm previous observations that over half the urinary coproporphyrin is liable to be excreted as a precursor; they are compatible with the thesis that all the urinary porphyrin is excreted in this way and part of it is converted to free porphyrin too rapidly to be recognized as precursor.

Biliary excretion in anaesthetized rabbits

The results of experiments in which the biliary and, in most cases, the urinary excretions of coproporphyrin were measured are shown in Table 1. Most of the experiments were performed before the importance of the precursor was recognized. The extractions were therefore performed by method *A* −, but they were carried out particularly slowly, in the intervals of attending to the experimental animal, so that most of any precursor present probably changed to the free porphyrin and was recovered as such. In later experiments, including those on rabbits 103 and 116 shown in the table and some on bile obtained from other anaesthetized rabbits, the full extraction procedure *A* was followed. When bile was extracted, a little additional porphyrin was always found in the ether allowed to stand until the following day, but in no case did the amount recovered represent more than an additional 0·5 μg./hr. It does not therefore appear that any serious error arose from the neglect of the unconverted precursor in the earlier experiments. In

Table 1. *Biliary and urinary excretion of coproporphyrin III after intravenous injection into anaesthetized rabbits*

(In these experiments coproporphyrin was extracted by procedure *A* –.) (N.D. Not done. N.U. No urine found in bladder.)

Treatment	Normal						Lead-treated							
Duration of lead treatment (days)	—		—		—		189		94		89		323	
Rabbit no.	111		113		116		78		98		99		103	
Weight (kg.)	2·0		1·9		1·8		2·1		1·9		1·7		2·3	
Coproporphyrin III (100 μg.) injected at beginning of 3rd hr.	No		Yes		Yes		No		Yes		Yes		Yes	
	(ml.)	(μg.)	(ml.)	(μg.)	(ml.)	(μg.)	(ml.)	(μg.)	(ml.)	(μg.)	(ml.)	(μg.)	(ml.)	(μg.)
Bile vol. and coproporphyrin content:														
1st hr. of collection	10·5	N.D.	10·3	1·3	10·4	1·1	7·3	1·1	7·2	1·0	9·3	4·8	6·1	1·1
2nd hr. of collection	7·2	2·6	7·9	1·3	4·8	0·2	6·5	1·3	6·0	0·4	4·2	5·1	6·5	1·0
3rd hr. of collection	7·4	N.D.	5·9	25·5	5·6	20·8	6·0	1·4	4·9	17·8	3·8	20·0	9·2	36·0
4th hr. of collection	7·9	1·4	6·4	19·6	3·8	20·3	6·9	2·1	3·8	9·0	3·1	15·9	9·4	27·6
5th hr. of collection	7·9	N.D.	6·1	21·0	0·5	1·2	8·2	1·6	3·1	6·8	Died		8·4	10·3
6th hr. of collection	3·4	1·0	5·9	14·0	0·6	1·1	11·7	1·7	2·2	4·3			8·2	6·5
Total in 6 hr.	44·3	10·0*	42·5	82·7	25·7	44·7	46·6	9·2	27·2	39·3	(20·4)	(45·8)	47·8	82·5
Urine vol. and coproporphyrin content:														
Total in 6 hr.	N.D.		37	7·5	39	1·7	20	27·0	N.D.		N.U.		9	25·0

* Estimated from average of available results.

order that the results may be comparable, even when additional precursor was looked for, only the quantities obtained in the first portion are given in Table 1.

Coproporphyrin was administered intravenously 2 hr. after beginning the collection of bile. An estimate of the normal biliary excretion of coproporphyrin under the conditions of these experiments can be based on all the results obtained in the 2 hr. before coproporphyrin was given. For five normal rabbits the mean excretion was $1{\cdot}3 \pm 0{\cdot}6$ (S.E.) μg./hr. and, for six lead-treated rabbits, $1{\cdot}7 \pm 1{\cdot}1$ (S.E.) μg./hr. (These figures include values for some rabbits not shown in Table 1.) This difference could easily occur by chance ($t = 0{\cdot}42$; $0{\cdot}7 > P > 0{\cdot}6$). The samples are small, but show that there is not a consistently increased amount of coproporphyrin in the bile of lead-poisoned rabbits comparable to the greatly increased urinary coproporphyrin.

When no coproporphyrin was administered, the rate of coproporphyrin excretion in the bile remained fairly steady. When coproporphyrin (100 μg.) was injected intravenously at the beginning of the third hour, the rate of excretion in the bile rose to 18–36 μg./hr. and then declined gradually, so that 35–75 % of the administered material was accounted for in the 4 hr. after injection. This was equally true of the normal and the lead-treated rabbits. In the two normal rabbits which survived for the whole period, 83 and 45 μg. respectively of coproporphyrin were recovered from the bile and in the two which had received lead, 83 and 39 μg. No excess of coproporphyrin, other than that expected in lead-treated animals, was found in the urine of any of the rabbits. While the close similarity of the biliary figures is presumably fortuitous, there is no evidence of an inability of the liver to excrete the administered material, or of a difference in the fate of coproporphyrin in the normal and the lead-treated rabbit.

All the porphyrins recovered from bile (from normal and lead-treated rabbits before and after coproporphyrin was injected) ran on paper with the same R_F as standard coproporphyrin III. The amounts present before coproporphyrin was injected were so small that if three- and five-carboxyl porphyrins had been present in the same proportion to coproporphyrin as they have been found in the urine of lead-treated rabbits, they would almost certainly not have been detected. The porphyrins recovered from the urine of these rabbits also ran on paper as coproporphyrin, and a trace of a three-carboxyl porphyrin was found in the urine of lead-treated rabbit 103.

Recovery of coproporphyrin from the urine after intravenous injection into unanaesthetized rabbits

In order to exclude abnormalities due to the anaesthesia and the operative procedure of the experiments on biliary excretion, coproporphyrin (100 μg.) was administered also to two conscious normal rabbits and to two conscious lead-treated rabbits. In order to obtain a free flow of urine, water was given by stomach tube in two doses of 50 ml./kg. 1 hr. apart and 1–2 hr. before injecting the coproporphyrin. The experiment was performed on a cross-over design, one rabbit of each pair receiving coproporphyrin and the other 0·15 M-sodium chloride on one day and vice versa on another day 3 or more days later. Urine was collected by suprapubic pressure at 30 min. intervals during the diuresis, but no appreciable difference was observed in the rate of flow of urine with or without administered coproporphyrin. In the normal rabbits there was no increase in the amount of coproporphyrin in the urine after its administration (Table 2). In the lead-treated rabbits the irregular but high rate of excretion made large differences in the total amount recovered insignificant. One rabbit (126) excreted less coproporphyrin in the 24 hr. after its administration than after the administration of saline, and the other rabbit (140) excreted more. However, the latter rabbit had been excreting still higher amounts of coproprphyrin in the 2 days immediately before the coproporphyrin was injected (996 μg. in 48 hr.), so the results do not suggest that there was any substantial urinary excretion of the administered porphyrin. Faeces were collected from these rabbits for 3 days after the injections. In each case more coproporphyrin was found in the faeces of the rabbit which had received coproporphyrin, but the quantities of faeces varied so greatly that the significance of this finding is doubtful.

In addition, coproporphyrin III was administered to some normal and some lead-treated rabbits without additional water, and the urine was collected over periods of 3 days. The amounts of porphyrin recovered did not show any increase after coproporphyrin was injected. The mean output of the normal rabbits in these experiments was unusually low (less than 1–2 μg./day) and it is possible that the porphyrin was decomposing during the period of collections: Schwartz *et al.* (1951) have shown that this occurs in acid urines, and the pH of these samples was not recorded. It is unlikely that large differences in urinary coproporphyrin concentration were obliterated by such deterioration, so the experiments provided some confirmation of the results obtained during diuresis on fresh samples.

Table 2. *Urinary volume and coproporphyrin content after intravenous injection of coproporphyrin III into unanaesthetized rabbits*

(Coproporphyrin was extracted by procedure *B*.)

Treatment	Normal rabbits							
Material injected	Saline				100 μg. coproporphyrin			
Rabbit no.	113		114		113		114	
Weight (kg.)	2·0		1·8		2·0		1·8	
Experimental day	1		2		2		1	
Urinary volume and coproporphyrin content:	(ml.)	(μg.)	(ml.)	(μg.)	(ml.)	(μg.)	(ml.)	(μg.)
0–4 hr. after injection	115	3·4	102	5·9	105	15·0	144	2·6
4–22 hr. after injection	151	8·8	115	18·0	97	8·3	115	8·5
Total	266	12·2	217	23·9	202	23·3	259	11·1

Treatment†	Lead-treated rabbits							
Duration of lead treatment (days)	66–73							
Material injected	Saline				100 μg. coproporphyrin			
Rabbit no.	136		140		136		140	
Weight (kg.)	1·9		2·2		1·9		2·0	
Experimental day	1		2		2		1	
Urinary volume and coproporphyrin content:	(ml.)	(μg.)	(ml.)	(μg.)	(ml.)	(μg.)	(ml.)	(μg.)
0–4 hr. after injection	94	28	109	69	166	50	36	128
4–22 hr. after injection	78	131	25	112	4	18	69	216
Total	172	159	134	181	170	68	105	344

Incidental observations on the effects of administering coproporphyrin

No abnormal behaviour was observed in the conscious rabbits after intravenous injection of 100 μg. of coproporphyrin. In the anaesthetized rabbits, there was usually a transient rise of blood pressure of 10–20 mm. Hg, which could be closely imitated by the injection of the same volume of 0·15 M-sodium chloride solution at the same rate. In one rabbit (113) the responses of the blood pressure to adrenaline, acetylcholine and nicotine were examined before and twice after giving coproporphyrin, and showed no appreciable change. Intestinal motility was not visibly altered in one normal rabbit (not among those included in Table 1), in which the intestines were exposed during and after the porphyrin injection. No other effects were observed. These observations do not support the suggestion that coproporphyrin is responsible for some of the symptoms of lead poisoning (Schreus & Carrié, 1933), particularly as the doses used were a large fraction of the usual total daily excretion of lead-poisoned rabbits.

DISCUSSION

These observations on the precursor of coproporphyrin in the urine of rabbits treated with lead show that it behaves similarly to the precursor described by Saillet (1896) and others in other circumstances. Attempts to prevent the precursor from changing to the free porphyrin have not been effective and there is therefore no direct evidence about the proportion of the urinary coproporphyrin excreted as precursor. Reasons are given below for believing the proportion to be very high.

The value of the experiments in which coproporphyrin was administered to rabbits depends on the identity of the administered material and of the material subsequently recovered and estimated. The identification of the material which was injected depends on its source and method of isolation, on the appearance of crystals of its methyl ester and their melting point, on the chromatographic homogeneity of the ester and of the free porphyrin obtained by hydrolysis, and on the absorption spectrum of the porphyrin so obtained. All these indicate that it was predominantly coproporphyrin III, but do not completely exclude the presence of coproporphyrin I and conceivably of other as yet unrecognized porphyrins. A melting point of 143°, for instance, is consistent with the presence of up to about 10% of coproporphyrin I in the material (Jope & O'Brien, 1945). In view of the origin of the material there was unlikely to be more than this amount of the type I isomer present even before crystallizing the methyl ester (Schwartz & Zagaria, 1951), although some recent observations suggest that urine from humans exposed to lead may contain a higher proportion of coproporphyrin I (Kench, Lane & Varley, 1952). Porphyrins which were presumed to have three or five carboxyl groups were separated chromatographically, and no other ether-soluble porphyrins are known to occur in the urine in lead poisoning.

The identification of the material extracted from urine and bile and subsequently estimated, depends on the method of isolation, fluorescence, absorption spectrum and behaviour as a four-carboxyl porphyrin on paper chromatography. In all these respects it was identical with the material administered, so it seems probable that it was in fact the same substance.

In the experiments on biliary excretion the operative procedures and anaesthesia may have affected the metabolism of coproporphyrin. Under these conditions urine was being secreted and coproporphyrin was found in it in concentrations comparable to those observed without anaesthesia. There is therefore no reason to believe that the kidney was functioning abnormally in this respect. Bile was being secreted, and administered coproporphyrin appeared in it promptly and in high concentrations. Even if the administered coproporphyrin was distributed no more widely than in the plasma, its concentration would not have exceeded about 1 μg./ml., since the plasma volume in rabbits of the size used in these experiments is about 100–120 ml. (Courtice & Gunton, 1949; Armin, Grant, Pels & Reeve, 1952) and the dose given was 100 μg. Concentrations up to 5 times greater were found in the bile, so the liver was capable of concentrating coproporphyrin and it is unlikely that the operative procedures increased its ability to do so. Also, administered coproporphyrin did not appear in the urine whether the animals were anaesthetized or not, so it seems probable that in the conscious animal, also, the coproporphyrin was being excreted in the bile.

With allowance for the rate of secretion in animals to which no porphyrin was given, the quantity of coproporphyrin obtained from the bile varied from 35 to 75% of the quantity administered. At the end of the experiments the biliary excretion was still above its original level, so the whole dose might have been excreted in this way. It is also possible that a fraction of the dose was fixed or metabolized in the body, but there is no evidence that less was metabolized by the lead-treated rabbits.

Before any porphyrin was administered, the concentration of coproporphyrin in the bile was similar in normal and lead-treated rabbits. In each case the liver was able to remove coproporphyrin from the plasma, so it is probable that there was no excess

of coproporphyrin circulating as such in the plasma of the lead-treated rabbits. The excess of coproporphyrin found in the urine must therefore have been carried in the plasma in some other form which was secreted by the kidney and then changed more or less rapidly into coproporphyrin. The observations on the occurrence of a precursor are consistent with this hypothesis.

It follows that the precursor is not excreted to any great extent by the liver. If it was it would have been demonstrable as such or as an excess of coproporphyrin in the bile of lead-treated rabbits, and probably coproporphyrin III would be found in the faeces in lead poisoning. A little precursor was found in the bile in these experiments, and Watson *et al.* (1951) have reported its presence in human fistula bile; so some hepatic excretion is possible. Indeed, it is possible that all the coproporphyrin found in bile, as in urine, has been excreted as a precursor. This possibility does not affect the present argument, provided that the capacity of the liver to excrete precursor is limited.

If the present findings in rabbits and the interpretation placed on them may be extended to other species and other causes of increased coproporphyrin excretion, the amount of coproporphyrin in the urine reflects the concentration of circulating precursor, whereas surplus coproporphyrin as such in the circulation is removed by the liver and appears in the faeces, or at least in the bile. If this were so, shifts from urinary to faecal excretion would correspond to an increased formation of free coproporphyrin from precursor in the body. Obviously, considerably more evidence is desirable to justify such a generalization.

As far as lead poisoning is concerned, it appears that the metabolic abnormality is an over-production or under-utilization of the precursor or precursors of coproporphyrin III and not of coproporphyrin III itself. Progress on these lines depends on identifying the precursor, which has been suggested to be a metalloporphyrin (Gray, 1951; Eriksen, 1951) or reduced coproporphyrin or coproporphyrinogen (Watson *et al.* 1951). The experiments reported here do not throw fresh light on this question.

SUMMARY

1. Experiments have been performed to discover why coproporphyrin III appears in the urine, but not in the faeces, in lead poisoning.

2. It has been found that at least half the urinary coproporphyrin in lead-treated rabbits is formed from a precursor after the urine is passed.

3. Coproporphyrin III has been obtained from the urine of lead-treated rabbits, crystallized as its methyl ester, hydrolysed and injected in 100 μg. doses intravenously into conscious and anaesthetized normal and lead-treated rabbits.

4. No additional urinary excretion of coproporphyrin III has been found.

5. In anaesthetized rabbits, the mean rate of excretion of coproporphyrin in the bile was $1{\cdot}3 \pm 0{\cdot}6$ (S.E.) μg./hr. for normal rabbits and $1{\cdot}7 \pm 1{\cdot}1$ (S.E.) μg./hr. for lead-treated rabbits. When 100 μg. coproporphyrin were injected intravenously, this rose to 18–36 μg./hr., and 35–75% of the dose was accounted for in the 4 hr. after injection. No difference was apparent between the biliary excretion of the normal and that of lead-treated rabbits.

6. It is suggested that the coproporphyrin found in the urine in lead-treated rabbits is excreted entirely as a precursor, and that the metabolic disorder produced by lead is an over-production or failure of utilization of a precursor of coproporphyrin and not of coproporphyrin itself.

7. The administered coproporphyrin had no effect on the behaviour, blood pressure or water diuresis of either normal or lead-treated rabbits.

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The Preparation and Properties of β-Glucuronidase

5. SPECIFICITY

By G. A. LEVVY and C. A. MARSH
Rowett Research Institute, Bucksburn, Aberdeenshire

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Knowledge of the specificity of the enzyme β-glucuronidase present in animal tissues is far from complete. Masamune (1934) found that the enzyme would hydrolyse neither *laevo*menthyl α-D-glucuronide nor a variety of α- and β-D-glucosides. Phenyl β-D-glucoside was slowly hydrolysed by Masamune's enzyme preparation, but Graham (1946) obtained no hydrolysis of this compound with highly purified ox-spleen β-glucuronidase. Karunairatnam (1950) found that phenyl β-D-glucoside was hydrolysed by mouse-liver homogenates, but that this property was lost in the early stages of purification of β-glucuronidase. Further information on the specificity of this enzyme, not recognized as such by the original authors, is provided by the work of Karunairatnam & Levvy (1949) who, in their search for an efficient inhibitor, found that the rate of hydrolysis of phenyl β-D-glucuronide was unchanged in the presence of methyl α-D-glucoside, methyl α-D-mannoside, methyl β-D-glucoheptoside, methyl β-D-xyloside and methyl α-D-galactoside. Had any of these compounds been substrates for the enzyme, a fall in the rate of liberation of phenol might have been expected.

Meyer, Linker & Rapport (1951) found that β-glucuronidase hydrolysed the oligosaccharides which are the end products of the action of purified testicular hyaluronidase on hyaluronic acid. In the absence of adequate knowledge of the specificity of β-glucuronidase, this finding could not be put forward as additional proof of the structure of hyaluronic acid. Implicit in the use of β-glucuronidase as a hydrolytic agent in the study of urinary steroids is the assumption that all the steroid set free in this way is initially conjugated with glucuronic acid. Further information on its specificity would increase the value of β-glucuronidase as a biochemical reagent. The difficulty of preparing suitable test compounds has been largely overcome by the development of a method for the oxidation of hexosides to hexuronides with gaseous oxygen in the presence of platinum catalyst (Marsh, 1952: cf. Fernandez-Garcia *et al.* 1950; Mehltretter, Alexander, Mellies & Rist, 1951; Barker, Bourne & Stacey, 1951). The opportunity has been taken to study the action of mouse-liver β-glucuronidase on α- and β-glucuronides, galacturonides and mannuronides, and on a variety of other compounds.

EXPERIMENTAL

Materials. The following compounds were synthesized by methods given in the literature and were converted into the corresponding conjugated hexuronic acids (Marsh, 1952): (−)-menthyl α-D-glucopyranoside, methyl α- and β-D-galactopyranosides, methyl α- and β-D-mannopyranosides, and β-D-glucopyranose-1-phosphate. Biosynthetic α-D-glucopyranose-1-phosphate, presented by Dr R. J. Whelan, was likewise put through the oxidation procedure. It should be noted that β-D-glucuronic acid-1-phosphate has not been obtained pure. The glucuronic acid content of the specimen used here was 60% of theory for the tripotassium salt.

Methyl α-D-glucopyranoside, phenyl β-D-glucopyranoside and (−)-menthyl β-D-glucopyranoside were also prepared by standard methods. Methyl α- and β-D-glucofuranuronides were obtained in solution by treating the corresponding 3:6-lactones with dilute NaOH. Methyl 4-methyl D-glucopyranuronide (mixture of α and β forms) was obtained in solution from the amide by heating the latter with NaOH (Smith, 1951). This amide and the two glucofuranuronolactones were presented by Prof. F. Smith. Oxycellulose (polyanhydroglucuronic acid, Yackel & Kenyon 1942) was presented by Imperial Chemical Industries, Ltd. (Explosives Group). It had a COOH content of 22% (theoretical value for polyanhydroglucuronic acid, 26%), and was brought into solution by careful addition of dilute NaOH, so that the pH never exceeded 7.

The standard substrates, phenolphthalein, phenyl and (−)-menthyl β-D-glucuronides, were biosynthetic.

Enzyme preparation. Unless stated to the contrary, the enzyme was prepared as follows. Mouse liver (1–2 g.) was homogenized in water and made up to a final volume of 9 ml.

After adding 1 ml. M-acetate buffer, pH 5·2, the homogenate was incubated at 37° for 30–60 min., and centrifuged at 1500 *g* for 15 min. The supernatant was decanted and to it was added an equal volume of saturated $(NH_4)_2SO_4$ solution. Precipitated protein was separated by centrifuging at 1500*g* for 15 min., and taken up in a convenient volume of water.

For measuring the liberation of reducing sugar, and for certain other experiments, the preparation of the enzyme was modified as follows. The homogenate was incubated at pH 5·2 for 4 hr., and the limits of precipitation with $(NH_4)_2SO_4$ were narrowed from 0 and 50% to 31 and 50% saturation. The final enzyme solution was dialysed for 48 hr. at 4° before use.

Measurement of enzyme activity. Three methods were used. (1) The liberation of phenolphthalein from 0·00063 M-phenolphthalein glucuronide in acetate buffer, pH 5·2, in 1 hr. was measured by a modification (Levvy, 1952) of the method of Talalay, Fishman & Huggins (1946). (2) The liberation of phenol from 0·015 M-phenyl glucuronide in citrate buffer, pH 5·2, in 1 hr. was measured by the method of Kerr, Graham & Levvy (1948), but using Ilford no. 608 red filter in the Spekker absorptiometer instead of the blue filter recommended in the original procedure. (3) Liberation of reducing sugar from various possible substrates during 2 hr. incubation at 37° in acetate buffer, pH 5·2, was measured by the method of Levvy (1946).

RESULTS

Effects on the hydrolysis of known substrates. All the compounds were examined for their effects on the hydrolysis of phenolphthalein glucuronide by glucuronidase, and the results are shown in Table 1. As shown by the action of phenyl β-glucuronide, the release of phenolphthalein should be slowed in the presence of an alternative substrate for the enzyme. This test for possible substrates has the advantage of being less dependent on the purity of the enzyme preparation than direct measurements of hydrolysis, but it does not discriminate between 'alternative substrate' and 'enzyme inhibitor'.

The galacturonides, mannuronides, glucofuranuronides and the 4-methylglucopyranuronide had no effects of any kind on the release of phenolphthalein and were ruled out as possible substrates, as were also the galactosides, mannosides, glucose-1-phosphates and two of the glucosides.

Of the compounds which changed the rate of hydrolysis of phenolphthalein glucuronide, oxycellulose caused a fall which, on the basis of the glucurone content, was equal to that produced by glucuronic acid under identical conditions of test (see Levvy, 1952). On dialysis, the solutions of oxycellulose lost most of their depressant effect, but only 20% of the solid present in solution was found to be dialysable. The oxycellulose solutions were made up either to pH 5, in which case they were buffered with acetate and dialysed against the same buffer, or to pH 7, in which case the unbuffered solution was dialysed against water. Similar results were obtained in the two cases.

Table 1. *Effects of possible alternative substrates on the hydrolysis of* 0·00063 M-*phenolphthalein β-D-glucuronide by mouse-liver glucuronidase*

Compound	Final concentration (M)	Change in rate of phenolphthalein liberation (% control)
(−)-Menthyl α-D-glucuronide	0·01	+22*
(−)-Menthyl β-D-glucuronide	0·01	+ 5*
Phenyl β-D-glucuronide	0·01	−16
Methyl 4-methyl-D-glucuronide	0·001	0
Methyl α-D-glucofuranuronide	0·0005	0
Methyl β-D-glucofuranuronide	0·001	0
α-Glucuronic acid-1-phosphate	0·01	−48*
β-Glucuronic acid-1-phosphate	0·001†	−26*
Methyl α-D-galacturonide	0·01	0
Methyl β-D-galacturonide	0·01	0
Methyl α-D-mannuronide	0·01	0
Methyl β-D-mannuronide	0·01	0
(−)-Menthyl α-D-glucoside	0·001	+4
Methyl α-D-glucoside	0·01	0
(−)-Menthyl β-D-glucoside	0·0025	+10
Phenyl β-D-glucoside	0·01	0
α-Glucose-1-phosphate	0·01	0
β-Glucose-1-phosphate	0·01	0
Methyl α-D-galactoside	0·01	0
Methyl β-D-galactoside	0·01	0
Methyl α-D-mannoside	0·01	0
Methyl β-D-mannoside	0·01	0
Oxycellulose (freshly prepared solution)	0·01‡	−49
Oxycellulose (solution dialysed for 48 hr.)	0·01‡	− 6
Oxycellulose (48 hr. old solution)	0·01‡	−42

* Enzyme precipitated between 31 and 50% saturation with ammonium sulphate and final solution dialysed.
† Calculated on glucuronic acid content. ‡ Calculated as glucurone (mol.wt. 176) before dialysis.

The depressant effects of the two glucuronic acid-1-phosphates on the hydrolysis of phenolphthalein glucuronide were consistent with the possibility that these compounds were substrates for the enzyme, but a number of other possibilities are considered later.

Activation of the hydrolysis of phenolphthalein glucuronide was observed in every experiment with menthyl α-glucuronide, varying somewhat in degree with different enzyme preparations. Activation was obtained with both menthyl glucosides and with menthyl β-glucuronide. With the β-glucuronide, activation of the enzyme apparently overcame the expected effect of substrate competition on the rate of liberation of phenolphthalein. Control experiments showed that neither of the menthyl glucuronides catalysed the decomposition of phenolphthalein glucuronide in absence of enzyme. Activation of the enzyme by menthol derivatives did not appear to be a surface effect since the surface-active agent, Triton X-100, did not activate the preparations used in the present experiments (Walker & Levvy, 1951). Mouse-liver glucuronidase can be separated into two fractions with pH optima at 4·5 and 5·2 respectively (Kerr, Campbell & Levvy, 1949). The activity of the mixture at a given pH is the sum of the activities of the two fractions at that pH. With a given enzyme preparation, activation by menthyl α-glucuronide was as great at pH 4·5 as at 5·2, which appeared to exclude the possibility of a change in the pH-activity curve.

Release of phenol from phenyl β-glucuronide was likewise accelerated by menthol derivatives, as shown in Table 2. On increasing the concentration of menthyl β-glucuronide, however, the effect of substrate competition became obvious. The affinities of phenyl and *laevo*menthyl β-glucuronides for the active enzyme centre, it should be noted, are similar, whilst that of phenolphthalein glucuronide is much greater (Karunairatnam & Levvy, 1949).

Table 2. *Effects of menthol derivatives on the hydrolysis of* 0·015 M *-phenyl* β-D *-glucuronide by mouse-liver glucuronidase*

Compound	Final concentration (M)	Change in rate of phenol liberation (% control)
(−)-Menthyl α-D-glucuronide	0·005	+19
(−)-Menthyl β-D-glucuronide	0·01	−20
(−)-Menthyl β-D-glucuronide	0·001	+ 3
(−)-Menthyl β-D-glucoside	0·0005	+ 6

The liberation of reducing sugar. Measurements were made of the liberation of reducing sugar from menthyl α-glucuronide and from α- and β-glucuronic acid-1-phosphate. For purposes of comparison, hydrolysis of menthyl β-glucuronide by the various enzyme preparations was also followed. Results of representative experiments are shown in Table 3. Menthyl α-glucuronide was neither a substrate for the enzyme, nor did it have any significant effect on the rate of hydrolysis of the β-anomer. Activation of the enzyme was presumably already at a maximum in the presence of the latter alone.

Neither α- nor β-glucuronic acid-1-phosphate yielded any reducing sugar after 2 hr. incubation at pH 5·2 in absence of enzyme, but both were hydrolysed in its presence. The glucuronic acid formed was insufficient to have accounted for the effects of the phosphates on the hydrolysis of phenolphthalein glucuronide (Table 1). Hydrolysis of the phosphates could have been due to β-glucuronidase or to a phosphatase in the preparation. Discrimination between these possibilities was made by the use of

Table 3. *Liberation of reducing sugar from various possible substrates by mouse-liver glucuronidase*

(Enzyme precipitated between 31 and 50% saturation with ammonium sulphate and final solution dialysed. Results expressed as glucuronic acid liberated in 0·8 ml. incubation mixture.)

Exp. no.	Substrate	Inhibitor	Glucuronic acid liberated (μg.)	Inhibition (%)
1	0·01 M-Menthyl β-glucuronide	None	69	—
	0·005 M-Menthyl α-glucuronide	None	0	—
	0·01 M-Menthyl β-glucuronide	0·005 M-Menthyl α-glucuronide	67	3
2	0·01 M-Menthyl β-glucuronide	None	129	—
	0·01 M-Menthyl β-glucuronide	0·0001 M-Saccharo-1:4-lactone	8	94
	0·01 M-α-Glucuronic acid-1-phosphate	None	27	—
	0·01 M-α-Glucuronic acid-1-phosphate	0·01 M-NaF	20	26
	0·01 M-α-Glucuronic acid-1-phosphate	0·0001 M-Saccharo-1:4-lactone	26	4
3	0·01 M-Menthyl β-glucuronide	None	113	—
	0·01 M-Menthyl β-glucuronide	0·0001 M-Saccharo-1:4-lactone	4	96
	0·002 M-β-Glucuronic acid-1-phosphate*	None	37	—
	0·002 M-β-Glucuronic acid-1-phosphate*	0·01 M-NaF	27	27
	0·002 M-β-Glucuronic acid-1-phosphate*	0·0001 M-Saccharo-1:4-lactone	6	84

* Concentration calculated on glucuronic acid content.

saccharo-1:4-lactone, a powerful inhibitor for β-glucuronidase (Levvy, 1952). As shown in Table 3, the concentration of the lactone chosen caused almost complete inhibition of the hydrolysis of menthyl β-glucuronide. Inhibition of the hydrolysis of β-glucuronic acid-1-phosphate was also marked, and this compound must therefore be regarded as a substrate for β-glucuronidase. The lactone had no significant effect on the rate of hydrolysis of α-glucuronic acid-1-phosphate. Hydrolysis in this case cannot therefore have been due to β-glucuronidase. The α-phosphate must have acted as an enzyme inhibitor, in the ordinary sense of the word, in the experiments with phenolphthalein glucuronide (Table 1).

Hydrolysis of both glucuronic acid-1-phosphates was slightly inhibited by fluoride, which does not inhibit β-glucuronidase (Karunairatnam & Levvy, 1949), suggesting that a phosphatase was present in the enzyme preparations.

Resynthesis of glucuronides. Storey (1950) has suggested that the synthesis of a glucuronide in the animal body may occur by condensation of the aglycone with glucuronic acid-1-phosphate (anomer unspecified) under the influence of a phosphorylase. It could also conceivably occur under the influence of β-glucuronidase, acting as a transferase. In the mouse, glucuronide synthesis occurs predominantly in the liver (Karunairatnam, Kerr & Levvy, 1949). The possibility had to be considered that resynthesis of phenolphthalein glucuronide might have occurred in the experiments with the glucuronic acid-1-phosphates shown in Table 1. The effects of the phosphates on the synthesis of *o*-aminophenyl glucuronide by mouse-liver preparations were therefore studied by the method of Levvy & Storey (1949).

Mouse-liver homogenates were variously prepared in water, 0·9% (w/v) sodium chloride, 1·1% (w/v) potassium chloride and bicarbonate Ringer solution. In no instance did incubation of the homogenate with α- or β-glucuronic acid-1-phosphate (0·001 and 0·005M) and *o*-aminophenol (0·0025%) in bicarbonate Ringer solution give detectable synthesis of *o*-aminophenyl glucuronide. Addition of 0·01M-sodium fluoride or 0·0002M-saccharo-1:4-lactone, or both, to the incubation mixture did not lead to synthesis. In the synthesis of *o*-aminophenyl glucuronide by mouse-liver slices, addition of α- or β-glucuronic acid-1-phosphate (0·001 and 0·005M) caused no stimulation, nor did these compounds overcome the inhibition produced by anaerobiosis (Storey, 1950).

DISCUSSION

The synthesis of *laevo*menthyl β-D-glucuronide, identical with the biosynthetic compound (Marsh, 1952), has afforded final proof that this compound and presumably other urinary glucuronides are β-D-glucopyranuronides. On the basis of the present work it would appear that mouse-liver glucuronidase is specific for this group of compounds, including β-D-glucopyranuronic acid-1-phosphate. It is remarkable that methyl β-D-glucofuranuronide was neither a substrate nor an inhibitor for the enzyme, since saccharo-1:4-lactone with a similar ring structure is a powerful competitive inhibitor (Levvy, 1952).

Oxycellulose on a weight basis was about as effective as free glucuronic acid in slowing the hydrolysis of phenolphthalein glucuronide by the enzyme, but in the former case the effect was largely associated with dialysable material in the solution, which amounted to only 20% of the total solid. The net effect was too great to be accounted for in terms of free glucuronic acid present as impurity in the oxycellulose solution. It is considered likely that the action of oxycellulose was due to a mixture of oligosaccharides composed largely of β-linked glucuronic acid residues. Since oxycellulose is unstable to alkali it was found impossible to apply the measurement of reducing sugar, to decide whether any hydrolysis occurred in the presence of the enzyme.

Depression of the enzymic hydrolysis of phenolphthalein glucuronide by α- or β-glucuronic acid-1-phosphate was open to a variety of explanations. The phosphate might have been an alternative substrate for the enzyme, or it might have been an enzyme inhibitor in the usual sense of the word. Hydrolysis, either spontaneous at the pH of enzyme assay, or under the action of some other enzyme than β-glucuronidase, might have yielded sufficient glucuronic acid to have caused marked inhibition. Lastly, resynthesis of phenolphthalein glucuronide might have occurred, in accordance with the theory of Storey (1950). Eventually it was found that β-glucuronic acid-1-phosphate was a substrate for β-glucuronidase and that the α-anomer inhibited the enzyme, although both compounds were slowly hydrolysed at pH 5·2 by a phosphatase in the glucuronidase preparations.

It should not be concluded that the experiments dealing with glucuronide synthesis disprove Storey's theory that glucuronic acid-1-phosphate is concerned in this process in the animal body. Some unknown factor may have been missing or deficient in the experiments with liver homogenates (Dutton & Storey, 1951). In the experiments with liver slices, on the other hand, the glucuronic acid-1-phosphates may not have penetrated the cell membrane. The results of our experiments do, however, make it very unlikely that any synthesis of phenolphthalein glucuronide from phenolphthalein can have occurred in the presence of β-glucuronidase preparations and α- or β-glucuronic acid-1-phosphate.

SUMMARY

1. On the basis of its action on a number of conjugated hexuronic acids and other compounds, it is concluded that mouse-liver glucuronidase is specific for β-D-glucopyranuronides, including β-D-glucopyranuronic acid-1-phosphate.

2. Derivatives of *laevo*menthol caused slight activation of glucuronidase.

3. Mouse-liver preparations did not catalyse the condensation of *o*-aminophenol with α- or β-glucuronic acid-1-phosphate.

The authors are indebted for various materials to the donors mentioned in the text, and to Dr P. G. Walker for assistance with many of the experiments on glucuronide synthesis.

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The Mechanism of the Action of Uricase

BY RONALD BENTLEY* AND A. NEUBERGER
National Institute for Medical Research, Mill Hill, London, N.W. 7

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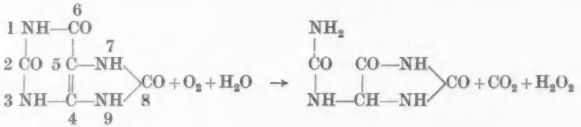
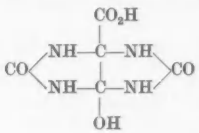
The enzyme uricase was considered formerly to oxidize uric acid with quantitative formation of allantoin, carbon dioxide and hydrogen peroxide (Wiechowski, 1907; Batelli & Stern, 1909; Keilin & Hartree, 1936*a*; Holmberg, 1939; Davidson, 1942), as shown in the following equation:



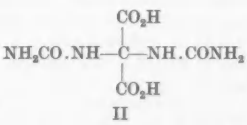
However, it was shown by Felix, Scheel & Schuler (1929) that under some conditions oxygen uptake was more rapid than carbon dioxide production, and Schuler (1932) found that at pH 8·9 the yield of carbon dioxide was only 17·8% of that expected from the oxygen consumption. These findings suggested that the first product of the enzymic oxidation was not allantoin, and the pH changes during oxidation indicated that the primary product was a substance of greater acid strength than uric acid. Indications of the structure of this first product were obtained in experiments in which uric acid was oxidized with permanganate. Fischer & Ach (1899) had found that oxidation of 1-methyl- and of 7-methyl-uric acid yields the same product, 3-methylallantoin, whilst 3-methyl- and 9-methyl-uric acid give 1-methylallantoin. Behrend (1904) pointed out that these findings can be explained by assuming that the first product of oxidation of uric acid by permanganate is a symmetrical compound, and he suggested that it possessed the structure I. This substance which Behrend was not able to isolate was named hydroxyacetylene diurein

* Present address: Department of Biochemistry, Columbia University, College of Physicians and Surgeons, New York.

carboxylic acid (HDC).* Later, Schuler & Reindel (1932) obtained HDC, in the form of a trisilver salt, by chemical oxidation of uric acid; these authors also observed that the free acid was unstable and was rapidly converted to allantoin and carbon dioxide. Finally, Schuler & Reindel (1933) succeeded in obtaining a silver salt with similar properties by oxidation of uric acid by uricase. It was concluded that the primary product of enzymic oxidation of uric acid was the symmetrical HDC (I).



I



II

Recently, the nature of the primary product of the enzymic oxidation was reinvestigated by Klemperer (1945) and by Praetorius (1948). Klemperer confirmed the earlier observations that with many buffer systems carbon dioxide production was smaller than calculated from the rate of disappearance of uric acid. From the amounts of nitrogen liberated under different conditions on treatment with nitrous acid, he concluded that the primary product is an unknown and unstable substance which gives rise to either HDC (I), uroxanic acid (II) or allantoin. Praetorius (1948) measured the changes in absorption of light which occurred during the oxidation of uric acid by uricase, using different buffer systems. He concluded that the primary, very unstable, product has maximum absorption in the long-wave ultraviolet (300–330 mμ.). This first product could then be converted either to a second product with a maximum of absorption at about 260 mμ. giving rise to allantoin or be transformed to HDC and further to uroxanic acid. Further evidence for the existence of a symmetrical intermediate in the chemical oxidation of uric acid and also in the oxidation occurring in the whole animal was provided by the use of ^{15}N-labelled uric acid (Brown, Roll & Cavalieri, 1947; Cavalieri & Brown, 1948).

In the work reported in this paper, the action of uricase has been studied in solutions containing $H_2{}^{18}O$ (gas phase $^{16}O_2$) and with systems in which $^{18}O_2$ was present in the gas phase. Further, [6-^{14}C] uric acid has been synthesized and used to study the source of the carbon dioxide evolved in the enzymic reaction.

* As pointed out to us by Dr R. S. Cahn, HDC should be called 5-hydroxy-3:7-diketo-2:4:6:8-tetra-aza*bicyclo* [3:3:0] octane-1-carboxylic acid.

EXPERIMENTAL

Materials

Uricase. Two preparations were used in these experiments: (*a*) a preparation made from pig-liver powder as described by Davidson (1942). Purification included dialysis against distilled water, extraction with phosphate buffer, pH 7·4, and solution in 0·1 M-borate, the final purification with $(NH_4)_2SO_4$ and acetone precipitation being omitted. The enzyme solution was a pale straw-coloured solution and when tested by Davidson's method had a specific activity of 32·8 μl./ml./min. It was free from catalase activity. (*b*) A second preparation was obtained through the courtesy of Dr E. Leone, Molteno Institute, Cambridge. This preparation was obtained by a method as yet unpublished and differed from other uricase preparations in not precipitating on dialysis against distilled water. It gave an O_2 uptake with uric acid of 270 μl./ml./hr.; dry wt., 2·5 mg./ml.

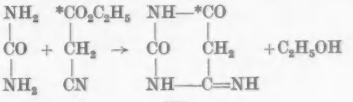
Catalase. This was made from horse red cells by the method of Bonnichsen (1947). The haematin content of the catalase solution was determined according to Keilin & Hartree (1936*b*). The solutions used were between 10^{-7} and 10^{-8} M.

Synthesis of [6-^{14}C] *uric acid*

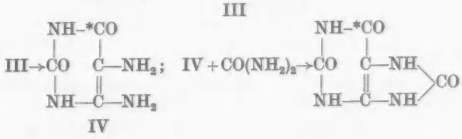
The first stages of the preparation of uric acid labelled in the desired position were based on the condensation of ethyl [*carboxyl*-^{14}C]cyanoacetate and urea to [4-^{14}C]6-aminouracil (III) (Traube, 1923) and the conversion of the latter by successive nitrosation and reduction to [4-^{14}C]5:6-diaminouracil (IV) (Traube, 1900). The experimental procedure actually used was the simplified method of Bogert & Davidson (1933). The diamino compound (IV) was condensed with urea (Johnson & Johns, 1914) to give [6-^{14}C] uric acid which was further purified by established methods and characterized by elementary analysis, absorption spectrum and by oxidation with uricase. The location of the labelled carbon atom is unambiguous as shown by the reactions employed (the labelled atom is indicated by an asterisk):

$$BrCH_2.{}^*COOH + KCN \rightarrow CN.CH_2.{}^*COOK$$

$$CN.CH_2.{}^*COOH + C_2H_5OH \rightarrow CN.CH_2.{}^*COOC_2H_5$$



III



IV

[*carboxyl*-^{14}C] Bromoacetic acid (162 mg.; 26 μc.), kindly supplied by Dr J. R. Catch, Radiochemical Centre, Amersham, was dissolved in water together with non-isotopic bromoacetic acid (14·34 g.). The solution was filtered and made up to 100 ml. To 95 ml. of this solution was added

sufficient 2N-KCN to bring the pH to approx. 8·5. Further KCN was added over a period of 3 hr. to maintain the pH at this value. Altogether 85 ml. 2N-KCN were required. N-HCl (50 ml.) was now added and the solution concentrated to dryness under reduced pressure. The solid was dissolved in ethanol (200 ml.) containing conc. H_2SO_4 (1 ml.) and the solution boiled under reflux for 3 hr. and then concentrated under reduced pressure. The residue was neutralized with 10% (w/v) $Na_2CO_3.10H_2O$. This solution was unfortunately left at a slightly alkaline pH for about 18 hr., partial saponification resulting. However, 1·19 g. ethyl [*carboxyl*-^{14}C] cyanoacetate distilling sharply at 97° (16 mm.) were recovered. This was combined with 9·55 g. of redistilled unlabelled cyanoacetate and converted to [4-^{14}C]5:6-diaminouracil described by Bogert & Davidson (1933). Yield was 8 g.

[4-^{14}C]5:6-Diaminouracil (4 g.) and urea (4 g.) were intimately mixed by grinding in a mortar and the mixture heated at 160° (bath temperature) for 1 hr. The mixture, which first liquefied and then solidified again, was dissolved in 0·5N-KOH and the filtered solution acidified with conc. HCl. The amorphous and impure uric acid was filtered off and redissolved in 0·5N-piperidine. An excess of solid NH_4Cl was added and ammonium urate filtered off after standing at 2° for 24 hr. The salt was converted to uric acid by digestion with 2N-HCl at 70°. Analysis indicated that this material was still somewhat impure. It was therefore dissolved in conc. H_2SO_4 and the solution filtered through glass wool. On careful addition of water crystalline uric acid slowly precipitated. The material was recrystallized from a large volume of boiling water. (Found: C, 35·4; H, 2·8; N, 33·0. Calc. for $C_5H_4O_3N_4$: C, 35·7; H, 2·4; N, 33·3%.) The ultraviolet absorption was measured on a solution containing 10 mg. uric acid/l. 0·001N-LiOH (pH approx. 11·0) using a Beckman spectrophotometer model DU; $\epsilon_{max.}$ was 12 300 at 294 mμ. and $\epsilon_{min.}$ was 2950 at 260 mμ. At pH 2·15 $\epsilon_{max.}$ was 10 900 at 284 mμ. These values agree well with those given by Stimson & Reuter (1943). On addition of uricase the ultraviolet absorption above 270 mμ. disappeared completely within 5 min. On measuring the oxygen absorption with uricase in a Warburg apparatus theoretical values were obtained. The specific radioactivity of the uric acid was $1·64 \times 10^{-3}$ μc./m-mole.

Isotope experiments

Reactions using isotopic oxygen were carried out in a closed system consisting of a 50 and a 25 ml. flask (both with B 19 ground-glass joints), connected by a suitable adaptor; this adaptor carried a vacuum stopcock through which the apparatus could be evacuated.

Reaction in $^{18}O_2$. A sample of the enzyme prepared according to Davidson (1942) (10 ml. in 0·1M-borate, pH 10) was pipetted into the 25 ml. flask; the larger flask contained 0·2M-borate (pH 10; 5 ml.) and a solution of uric acid in LiOH, prepared as described by Davidson (20 ml. containing 0·11 g. uric acid). The ground joints and stopcock were well greased and the contents of the flasks frozen in an ethanol-CO_2 bath. The system was evacuated, the stopcock closed, and the solutions allowed to thaw and de-gas. After freezing again, evacuation was completed. The reaction system was attached to an electrolysis apparatus (Bentley, 1949) in which $^{18}O_2$ was generated. The liquids were kept frozen during the electrolysis which was continued until approximately 15 ml. of $^{18}O_2$ (at N.T.P.) had been liberated.

The stopcock was closed, the reaction system detached from the vacuum line and the contents of the flasks thawed and mixed. The apparatus was shaken gently for 1 hr. in a bath at 37°, the flasks being submerged. The liquid was then all collected in the large flask and frozen in ethanol-CO_2. The residual gas was examined in the mass spectrometer. It contained only a trace of CO_2. Oxygen was plentiful and had atom % excess $^{18}O = 0·584$. Air was admitted, a second 25 ml. flask containing catalase solution (0·5 ml.) was attached and the H_2O_2 formed in the reaction was decomposed *in vacuo* using the technique previously described (Bentley & Neuberger, 1949). The oxygen produced had atom % excess $^{18}O = 0·571$.

The system was again brought to atmospheric pressure and a flask containing conc. H_2SO_4 was attached. The solutions were mixed *in vacuo* in the usual way: an abundant amount of CO_2 was produced which did not contain any excess ^{18}O above normal.

Reaction in $H_2{}^{18}O$. Uricase solution ('Davidson', 20 ml.) was treated with an equal volume of ice-cold, saturated $(NH_4)_2SO_4$. The precipitate was centrifuged in the high-speed centrifuge at 0° and redissolved in $H_2{}^{18}O$ (4 ml., atom % excess = 1·20). To this solution were added $Na_2B_4O_7.10H_2O$ (300 mg.), uric acid (50 mg.) and LiOH.H_2O (12·5 mg.). The mixture was shaken in a bath at 38° for 3 hr. (atmosphere of normal O_2). The O_2 uptake was approximately 5 ml. at N.T.P. The contents of the flask were then decomposed successively with catalase and conc. H_2SO_4 as previously described. O_2 from catalase decomposition: 0·000 atom % excess ^{18}O. CO_2 from H_2SO_4 decomposition: 1·18 atom % excess ^{18}O.

To check whether CO_2 would exchange under these experimental conditions, uric acid (25 mg.), LiOH.H_2O (6·5 mg.), $Na_2B_4O_7.10H_2O$ (150 mg.), and $NaHCO_3$ (5 mg.) in $H_2{}^{18}O$ (2 ml., atom % excess = 1·20) were shaken under the same conditions. The CO_2 obtained after treatment with conc. H_2SO_4 had 1·13 atom % excess ^{18}O.

Experiment with [6-^{14}C] *uric acid.* Uricase solution ('Leone's preparation', 23 ml.) was dialysed overnight against tap water at 0° and then against fresh distilled water at 0° for 2 hr. $Na_2B_4O_7.10H_2O$ (0·95 g.) was added to the dialysed enzyme solution, followed by a solution of [6-^{14}C] uric acid (75 mg., $1·64 \times 10^{-3}$ μc./m-mole) and LiOH.H_2O (20 mg.) in boiled-out water (5 ml.). The mixture was shaken for 2 hr. at 38° in an O_2 atmosphere. Conc. H_2SO_4 (3 ml.) was then run into the flask and the solution aspirated in a stream of CO_2-free N_2. CO_2 in the emergent gas was precipitated as $BaCO_3$; two successive precipitates were collected. They were centrifuged, washed with boiled-out distilled water and ethanol, and dried *in vacuo* over P_2O_5. For counting, they were resuspended in absolute ethanol and filtered using the apparatus and perforated disks described by Popják (1950). Sample *A* (2·5 mg.) contained $1·34 \times 10^{-3}$ μc./m-mole; sample *B* (7·4 mg.) contained $1·28 \times 10^{-3}$ μc./m-mole (both values are corrected to infinite thickness).

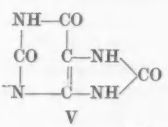
DISCUSSION

The experiments with labelled gaseous oxygen and oxygen-labelled water prove conclusively that uricase catalyses the transfer of hydrogen, or more probably of electrons, from uric acid to the oxygen of the gas phase. The prosthetic group of uricase is

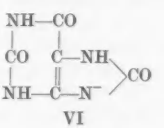
not known, but the general mechanism of the action of this enzyme resembles that of the flavin enzyme, notatin, which has been the subject of an earlier communication (Bentley & Neuberger, 1949). Electron transfer may occur in two distinct steps as has been postulated for all biological oxidations by Michaelis (1946). If this is the case, the electron acceptor O_2 is converted in the first step into the free radical O_2^- or, if a proton is taken up at the same time, into the free radical $HO_2^{\cdot}$. In the second step these radicals will be converted into hydrogen peroxide or one of its ions, HO_2^- or O_2^{2-}.

The finding that the carbon dioxide which arises in the oxidation of uric acid by uricase is derived from the carbon atom in position 6 of the uric acid molecule is not unexpected and does not by itself indicate how allantoin is formed from the primary oxidation product. However, the results obtained in the present work, together with the many observations made by other workers, suggest a probable mechanism for this rather complex reaction. Owing to the exchange reaction between carbon dioxide and water which took place under the experimental conditions, it was impossible to determine the source of the oxygen atoms of carbon dioxide.

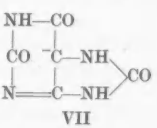
Uric acid is a dibasic acid with pK_1 5·4 and pK_2 10·6 (Johnson, 1952) and the actual substrate of uricase is therefore almost certainly either the mono-anion or, in view of the rather alkaline pH optimum (9·25; Keilin & Hartree, 1936*a*) of the enzyme, the di-anion. Which of the four hydrogen atoms of uric acid dissociate as protons in the first and second steps of the ionization is not certain; but Biltz & Herrmann (1921) deduced from a comparison of the ionization constants of various methyluric acids, that the imino-hydrogen atom in position 3 was the most acidic and that the hydrogen in position 9 had a slightly weaker tendency to dissociate. Later, Biltz (1936*a*, *b*) suggested that the order of acidities of the hydrogen atoms was $9>3>7>1$. Thus the mono-anion may be represented by one of the tautomeric formulae V or VI:



V

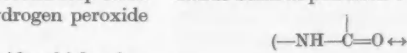


VI

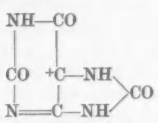


VII

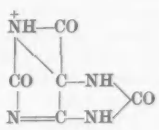
For each tautomeric form there are several resonance structures, some of which are derived from similar structures of the undissociated uric acid molecule. Resonance in the ion, however, is greater than in the acid, and the negative charge in the former is not localized on the nitrogen atom, but will be spread over adjacent carbon and oxygen atoms. One set of structures which is likely to make an important contribution contains the negative charge on the carbon atom in position 5; these structures, one of which is shown (VII), will be favoured by the polar character of the adjacent amide bond in positions 7 and 8



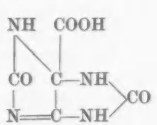
It is suggested that the actual substrate of uricase is a mono-anion represented largely by a structure such as VII or a di-anion carrying a second charge in another unspecified position. The enzyme is then assumed to catalyse either a simultaneous or successive transfer of two electrons to oxygen, giving as primary product a carbonium ion such as VIII. It is proposed that this transfer of electrons is the only step catalysed by the enzyme and that the further changes observed are purely chemical reactions arising from the reactivity of the intermediates, especially that of the carbonium ion structure.



VIII



IX



X

In a carbonium ion such as VIII carbon atom 5 is strongly electrophilic and will have a tendency to interact with the nitrogen atom in position 1 which is the only nucleophilic centre with which, for geometrical reasons, a bond can be formed. The resulting structure IX is unstable, largely owing to strain of the three-membered ring, and reaction with a hydroxyl ion will give X.

Rearrangements involving intermediate formation of carbonium ions are common in organic chemistry, but a particularly close analogy to the proposed series of reactions is afforded by the rearrangements of bicyclic α-amino ketones during Clemmensen reduction (Leonard & Wildman, 1949; Leonard & Ruyle, 1949). In these reactions a bond between a positively charged carbon and a tertiary nitrogen atom is formed, resulting in a transformation of a system containing two fused six-membered rings to one containing a five- and a seven-membered ring. The acid X, which may be the primary product

of Praetorius (1948), is likely to have some aromatic character owing to the presence of a double bond and the resonance of the amide bonds, and thus will show absorption of light in the ultraviolet range. Although the compound X is not symmetrical, the double bond is likely to shift readily to the other ring since the adjacent imino-hydrogen atom will be very mobile. It is further suggested that compound X may be transformed in three ways. It may give rise, by opening of one ring, addition of the elements of water, and loss of carbon dioxide, to allantoin, possibly involving another unstable intermediate; both rings may be opened with the addition of the elements of two water molecules giving uroxanic acid (II), or hydration may occur at the 3:4 double bond to give the symmetrical HDC (I).

This sequence of reactions explains satisfactorily most of the findings of other workers and is in accord with the results presented here. In the first place it is reasonable to assume that an enzyme catalyses one step in a reaction; the results obtained with ^{18}O clearly indicate that this step is a transfer of two electrons and this must yield a carbonium ion, if the mono-anion is the substrate, or a dipolar molecule of similar structure, if the di-anion were the substrate. The rearrangement of a six- or a five-membered ring invokes a mechanism accepted in the theory of organic reactions. The results of Felix *et al.* (1929) and those of Schuler (1932), Klemperer (1945) and Praetorius (1948) clearly indicate that the first product of uricase oxidation is not allantoin, but a compound still containing the same number of carbon atoms as uric acid. That this product is not HDC is suggested by the ultraviolet absorption data of Praetorius (1948) and also by the fact that this unstable product yields allantoin apparently more readily than HDC. It is therefore suggested that this substance has the structure X and that it can be converted to allantoin without preliminary hydration to HDC. The tautomeric shift of the double bond mentioned above explains the finding of Brown *et al.* (1947) that on feeding [1:3-^{15}N] uric acid to rats, the urinary allantoin is uniformly labelled both in the hydantoin and urea moieties; presumably the only reaction involved in this *in vivo* experiment was oxidation by uricase. An analogous mechanism involving X would also explain the similar uniform isotope distribution in allantoin obtained by the alkaline oxidation of [1:3-^{15}N] uric acid (Cavalieri & Brown, 1948).

The concept that the primary product of oxidation has a carbonium ion structure is likely to apply to many or most biological oxidations. In the special case of the oxidation of the urate ion by uricase the reaction is not complicated by the presence of a hydrogen atom on the reactive carbon centre; oxidation simply consists of a transfer of electrons. In most oxidations this electron transfer is accompanied by a proton transfer; thus the oxidation of an aldehyde to the corresponding acid may be written thus:

$$RCHO \rightarrow RC^{+}{=}O + H^{+} + 2\epsilon^{-}, \quad (1)$$

$$RC^{+}{=}O + OH^{-} \rightarrow RCOOH. \quad (2a)$$

This proposed mechanism also provides a reasonable explanation of the linkage of oxidation with formation of a labile phosphate bond of high energy, since the carbonium ion may react with a phosphate ion, instead of a hydroxyl ion:

$$RC^{+}{=}O + HPO_4^{2-} \rightarrow RC\begin{matrix}O\\ \diagdown\\ \diagup\\ OPO_3H^{-}\end{matrix}. \quad (2b)$$

In the reaction with water the high energy of the carbonium ion is dissipated as heat, whilst in the reaction with phosphate it is transformed into phosphate-bond energy. It is assumed that this reaction does not take place with free phosphate in solution, but with an ion which is adsorbed on the enzyme protein and is placed in a favourable geometrical position with regard to the substrate which is also combined with the enzyme.

A similar mechanism can also be suggested for the linkage between oxidative decarboxylation and phosphorylation:

$$RCO.COO^{-} \rightarrow RC^{+}{=}O + CO_2 + 2\epsilon^{-}, \quad (3)$$

$$RC^{+}{=}O + HPO_4^{2-} \rightarrow RC\begin{matrix}O\\ \diagdown\\ \diagup\\ OPO_3H^{-}\end{matrix}. \quad (4)$$

Such enzyme complexes containing labile phosphate bonds may transfer their phosphate to adenosinediphosphate or other substances.

SUMMARY

1. By using water and gaseous oxygen labelled with ^{18}O it is shown that the oxygen atoms of the hydrogen peroxide formed in the oxidation of uric acid by uricase are derived from molecular oxygen. It is concluded that uricase catalyses the transfer of two electrons from a urate ion to oxygen.

2. Uric acid labelled with ^{14}C in the 6 position was synthesized. It is shown that the carbon dioxide formed in the enzymic oxidation is derived from the labelled carbon atom.

3. On the basis of the results reported here and the work of others a mechanism of enzymic oxidation of uric acid is proposed which assumes that the enzyme acts on the anion transforming it into a carbonium ion with loss of two electrons. The further changes which are probably non-enzymic consist mainly of an electrophilic rearrangement characteristic of carbonium ion structures.

4. The possible role of carbonium ions as intermediates in biological oxidations and coupled phosphorylations is discussed.

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A Combustion Technique for the Assay of Tritium, ^{13}C and ^{14}C in a Single 10 mg. Sample of Biological Material

By R. F. GLASCOCK
National Institute for Research in Dairying, University of Reading

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Payne, Campbell & White (1952) have recently described their method for the quantitative combustion of 500–600 mg. samples of tritiated metabolites. This scale is dictated by the method they prescribe for the determination of tritium, namely the reaction of 100–200 mg. of water with aluminium carbide and the counting of the tritiomethane so produced in a proportional gas counter. As it is not always easy or even possible to isolate as much as 0·5 g. of a particular metabolite other workers may be interested to know that there was recently published (Glascock, 1951*a*, *b*) a method for determining tritium on the 5–10 mg. scale. Sufficient water for the determination of tritium by this method and sufficient carbon dioxide for the determination of both ^{13}C and ^{14}C are produced by the quantitative combustion of about 10 mg. of most organic substances. A method for doing this has been in routine use in this laboratory for the past two and a half years, and a description of it now seems appropriate in view of the communication by Payne *et al.* (1952). It is very much easier to burn 10 mg. quantitatively than 0·5 g. and thus obtain from 10 to 20 ml. of carbon dioxide. This is usually more than enough for the gas counting of several samples by the method of Brown & Miller (1947) and a few ml. can readily be spared for the mass spectrometer if ^{13}C is to be determined also. Then, if the compound is one which yields relatively little carbon dioxide, either a rather larger sample is burnt or the dose of ^{14}C is adjusted in advance so that smaller samples can be counted, thus leaving more for the spectrometer. The water produced usually amounts to about 10 mg. and it is treated with an excess of *n*-butyl magnesium bromide in a previously prepared 'Grignard tube' to yield *n*-tritiobutane almost quantitatively (Glascock, 1951*a*, *b*). If, for some reason, tritiomethane were preferred it could probably be prepared on this scale by using the appropriate Grignard reagent but butane has several important advantages. Firstly, it is an excellent counter gas and can be counted with high efficiency in the Geiger region of a suitable gas counter; hence the additional electronic equipment required for proportional counting as used by Payne *et al.* is unnecessary. Secondly, unlike methane,

butane is fully condensed at the temperature of liquid oxygen and samples of gas can be easily measured out and quantitatively transferred. In fact it is possible to measure out and transfer to the counter between 10 μl. and 10 ml. of gas, thus permitting the counting of samples of widely differing specific activities. Tritium is available in very high activities, much higher, for example, than is ^{14}C, and it is therefore very easy to arrange the dose so that 10 mg. samples of metabolite have a suitable activity.

standard CuO filling. It is maintained at a temperature of 600°, being switched on automatically in the morning, a few hours before work is due to start. The chilled traps, T_1 and T_2, consist of a spiral and a simple U-tube, respectively. The spiral comprises 50 cm. of 4 mm. bore tubing and condenses out the greater part of the combustion products, the rest being trapped in T_2.

The apparatus to the left of the line X–X is kept on open pumps and, by adjusting the stopcock S_{12}, O_2 is drawn through the combustion train from the reservoir R at about 50 ml./min. Thus the pressure in the chilled traps does not rise above about 2 mm. Hg. S_{12} is a 'control' stopcock, that

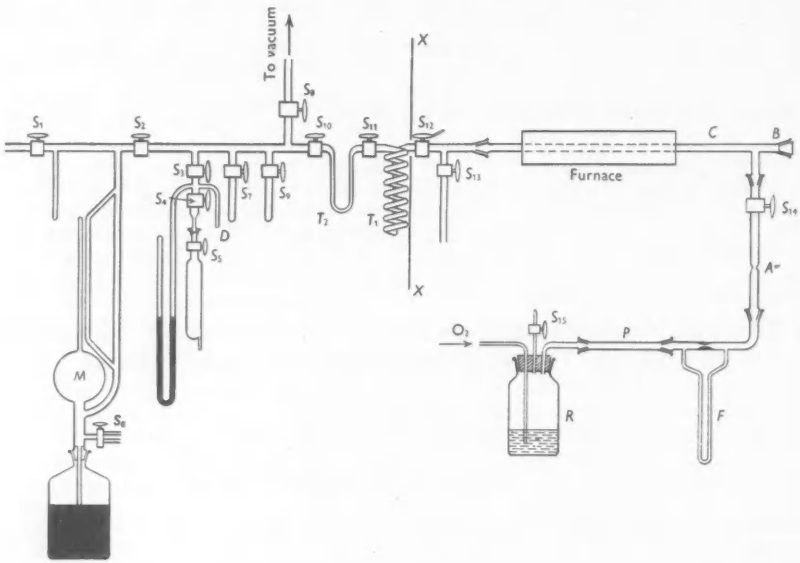

Fig. 1. Vacuum apparatus and combustion train. S_1 to S_{15} = stopcocks; M = McLeod gauge; C = part of combustion tube in which boat is placed; B = stoppered joint through which boat is introduced into combustion tube; T_1 = spiral trap; T_2 = U-trap; A = tube of Anhydrone and soda-asbestos; F = flowmeter; P = tube of platinized asbestos; R = oxygen reservoir.

EXPERIMENTAL

Apparatus. The combustion apparatus is shown in Fig. 1. It differs from the conventional micro-analytical apparatus in two important respects. Firstly, the products of combustion are not collected chemically, but are stripped from the O_2 stream in liquid-O_2-chilled traps (cf. Glascock, 1951*b*; Balmain, Folley & Glascock, 1951). Secondly, since a condensable component of a gas mixture is stripped out most efficiently at low pressures, the conditions are arranged so that the pressure in the traps is only a few mm. Hg.

The whole apparatus, with the exception of the quartz combustion tube, is made of Pyrex glass. The combustion tube itself is 50 cm. long with an internal diameter of 10 mm. and is fitted with *B* 10 ground joints as shown in Fig. 1. The 30 cm. of its length within the furnace are packed with the is, one with a long handle to the plug and shallow file cuts on opposite sides of the bore. It is a convenient device for controlling gas streams accurately at low rates and has been described before (Arrol & Glascock, 1948).

The O_2 is fed into the reservoir R from a cylinder, is roughly dried by bubbling through conc. H_2SO_4 and then runs mostly to waste through the stopcock S_{15}. The O_2 drawn into the combustion tube passes first through hot platinized asbestos P (to oxidize any combustible gases), a simple flow meter F, and a tube packed half with soda-asbestos and half with Anhydrone A. Chemical rather than physical purification of the gas is preferable since several chilled traps would be needed to strip out contaminating water and CO_2 at atmospheric pressure. When a combustion is not being carried out the O_2 stream is diverted to waste through the stopcock S_{13} thus passing through the hot combustion tube.

The sample is weighed into a porcelain boat and placed in that part, C, of the combustion tube which is outside the furnace. O_2 is passed through the tube and the sample heated gently in a luminous flame until it starts to burn. It is then heated strongly for 5 min. in a Meker burner to complete the combustion. At the end of this time, which is ample for the quantitative combustion of 10 mg. samples, the stopcock S_{14} is closed and the control stopcock S_{12} opened wide, thus evacuating the combustion tube and drawing residual gas through the traps. The control stopcock is then closed and the traps evacuated to a very low pressure (mercury-vapour pump).

In checking this method (see Tables 1 and 2) the criterion of quantitative combustion has been the yield of CO_2, which can be measured manometrically with an error not exceeding 1%. It has been found that more than 25 mg. cannot always be burnt quantitatively owing to too vigorous firing; mixing the sample with about its own weight of CuO catalyst, however, permits a more controlled combustion of up to 100 mg. The contents of the traps T_1 and T_2 are combined in T_2 by allowing the first to warm to room temperature with the stopcock between them open and, by raising the temperature of trap T_2 from $-183°$ to $-78°$ (dry ice and acetone), the CO_2 is sublimed away from the water and is transferred to the calibrated space between stopcocks S_3 and S_4, measured and stored until required in a side arm of which two, attached to stopcocks S_7 and S_9, are shown. Any traces of contaminating tritium activity are eliminated

Table 1. *Quantitative combustion of substances of known composition*

(All weighings carried out on Oertling air-damped aperiodic balance. 1 div. = 0·2 mg.)

Substance	Wt. combusted (mg.)	CO_2 at N.T.P. (ml.) Found	Calc.
Palmitic acid, $C_{16}H_{32}O_2$	10·4	14·50	14·50
Palmitic acid, $C_{16}H_{32}O_2$	8·6	11·80	12·00
Stearic acid, $C_{18}H_{36}O_2$	5·8	8·10	8·20
Stearic acid, $C_{18}H_{36}O_2$	8·0	11·05	11·30
Glucose, $C_6H_{12}O_6$	12·3	9·20	9·20
Glucose, $C_6H_{12}O_6$	13·4	10·00	10·00

Table 2. *Reproducibility of results*

(Sample combusted and assayed was calcium salts of mixed total fatty acids isolated from mammary-gland slices after incubation *in vitro* in bicarbonate-Ringer containing [^{14}C]glucose and doubly labelled acetate ($CH_2{}^3H.{}^{13}COONa$) as substrate. (Unpublished results of Balmain, Folley & Glascock.))

A. Triplicate combustions: yield of CO_2

Combustion no.	Weight combusted (mg.)	CO_2 obtained at N.T.P. Total (ml.)	(ml./mg. salt)
1	13·1	13·60	1·037
2	8·8	9·05	1·029
3	11·1	11·40	1·029

B. Assay of products (tritiobutane first being prepared from combustion water)

Gas assayed	Combustion no.	Vol. counted (ml. at N.T.P.)	Activity (counts/min.)	Specific activity (counts/min./ml.)
Butane	1	2·60	429	165·0
		3·10	500	161·0
	2	2·20	358	162·5
		3·15	504	160·0
	3	2·80	447	159·5
		2·50	399	159·5
Carbon dioxide	1	4·35	1883	433·0
		2·95	1297	440·0
	2	2·80	1187	424·0
		1·90	828	436·0
	3	0·88*	387	440·0
		3·70	1576	426·0

Gas assayed	Combustion no.		Total ^{13}C abundance (atom %)	Excess ^{13}C (atom %)†
Carbon dioxide	1	—	1·230	0·140
			1·229	0·139
	2	—	1·232	0·142
			1·231	0·141
	3	—	1·233	0·143
			1·228	0·138

* Measured in McLeod gauge.

† Experimental value for natural abundance of ^{13}C found to be 1·09 atoms %.

from the CO_2 before counting by adsorption on active charcoal at $-78°$ and desorption at 0°. Both processes take place very rapidly and cause negligible delay in the course of an assay. The water, which remains in trap T_2, is distilled *in vacuo* into a previously prepared 'Grignard tube' for the generation of butane and assay of tritium.

As the actual combustion takes only 5 or 6 min. the time taken for the assay of each sample depends chiefly on the speed of counting and the time needed to clear one batch of isotopic gases from the apparatus to make room for the next.

The presence of combined nitrogen in the sample interferes with the assay of none of the isotopes. Thus, separate samples of glyceryl tri-*p*-nitrobenzoate labelled with ^{14}C and tritium, respectively, have been successfully assayed.

Measurement of gas samples. In the calibrated space, S_3–S_4, volumes of gas between 1 and 15 ml. can be measured manometrically, with an error of less than 1%. In the McLeod gauge, M, volumes between 10 μl. and 1 ml. can be measured with about the same accuracy. The reservoirs of inactive butane and CO_2 (2 l. bulbs), not shown in the diagram, communicate with the space S_3–S_4 and are used to adjust the pressure of the gas in the counter to its working value after condensing in a measured sample of radioactive gas. In routine use it has been found that two samples usually give closely agreeing specific activities (see, for example, Glascock, 1951*a*, *b*; and Table 2). If the sample to be counted is less than 1 ml. it is convenient to fill the counter with inactive gas, count the background, and then, after re-attaching to the vacuum apparatus, add the sample to be counted. Counters filled with either CO_2 or butane have such good characteristics that the slight increase in pressure introduces no measurable error.

Elimination of contamination. There has been much reference in the literature to the errors arising from contamination of silica and glass apparatus by hydrogen isotopes. No such contamination has been detected after combustion of radioactive samples by the method and on the scale described in this paper. Thus no tritium or ^{14}C activity was found when a sample of inactive glucose was combusted and assayed immediately after a 12 mg. sample of fatty acid containing a total of 1150 counts/min. of tritium and 3300 of ^{14}C. This may be due partly to the fact that a stream of O_2 is continuously passed through the hot combustion tube whether it is in use or not. As an additional precaution the two traps are flamed out under high vacuum with the luminous flame of a hand torch between combustions.

DISCUSSION

Solid and gas counting: sample size. When the stable isotope ^{13}C is to be determined, combustion to carbon dioxide cannot be avoided since only gaseous substances can be assayed in the mass spectrometer. Compounds containing ^{14}C, however, can be counted directly either by means of a thin end-window counter or in a flow-type counter. Although the direct counting of organic substances is very convenient and widely used, the method suffers from certain disadvantages: firstly, not all compounds can be spread on a disk to form an even layer giving reproducible counting rates; secondly, some loss of accuracy is involved in comparing activities when the radioactive isotope is combined in compounds of markedly different chemical composition. Many workers therefore prefer to reduce all their organic samples to a common chemical form, usually barium carbonate. This is accomplished by combustion and absorption of the carbon dioxide in barium hydroxide. Its activity as measured with an end-window counter is compared with that of a standard barium carbonate sample of known activity which is counted at the same time.

An elegant method for counting very thin samples of fatty acids spread over a large area (from 5 to 30 mg. on 15 sq.cm.) by deposition from solution on a lens paper has been described by Entenman, Lerner, Chaikoff & Dauben (1949). This is applicable, however, only to certain substances, and for general work most workers prefer to count samples at infinite thickness, which for ^{14}C is about 20 mg./sq.cm. (Kamen, 1947), since this eliminates the correction for self-absorption and consequent errors. Disks of 2 or even 3 sq.cm. area are commonly used since the larger the disk the relatively smaller the 'edge effect'. Thus, allowing for transfer losses, it is inconvenient if less than 75–100 mg. of sample are available for solid counting at infinite thickness. This is to be contrasted with the 10 mg. which are sufficient for the combustion method described in this paper; for although 100 mg. of metabolite are easier to obtain than 0·5 g. it is often still more convenient if much less can be accurately assayed. For example, not more than about 80 mg. of mixed fatty acids may be obtainable from the liver of a single rat and it is sometimes necessary to fractionate this quantity into several components. Similarly, the quantity of fatty acids obtainable from 1 g. of mammary tissue used in a tissue-slice experiment is only 30–40 mg. and is too little to count conveniently in the solid state.

Counter efficiency: dose levels. For the counting of β-particles from ^{14}C the efficiency of a gas counter is at least 80%. The efficiency of an end-window counter mounted in the conventional lead castle (i.e. about 5 mm. from the source) is reduced by more than a factor of 10 as a result of geometry and absorption by the window and there is a further loss due to self-absorption by the sample. For example, it was found that a sample of fatty acid spread at infinite thickness on a disk of area 1 sq.cm. gave 100 counts/min. when measured with an end-window counter (EHM 2). The same sample combusted and the whole of the carbon dioxide introduced into a gas counter had an activity of 4200 counts/min. There is thus a gain in efficiency by a factor of 42. The corresponding gain for glucose is 56. The experimental results from which these values are calculated are given in Table 3.

If an organic compound labelled with ^{14}C has an activity at infinite thickness of A counts/min. it can

be shown that if the carbon in it were isolated and counted at infinite thickness it would have an activity of $100A/BC$ counts/min., where C is the percentage of carbon in the compound and B is the backscattering factor. For compounds containing only light elements B has the value of 1·04 (Calvin, Heidelberger, Reid, Tolbert & Yankwich, 1949), and for purposes of comparison of activities it can therefore be neglected. For compounds containing heavy elements, however, B is larger and must be taken into consideration. Thus the value for $BaCO_3$ is 1·30.

Workers who prefer to count uncombusted compounds at infinite thickness claim that this formula gives a measure of the specific activity of the carbon to within 5 or 6 % providing care is taken to spread the sample so that a uniform surface is obtained. Other workers find that the counting of uncombusted samples is subject to inaccuracies greater than this. For example, Abrams & Clark (1951) say, 'We have been able to get ^{14}C counts accurate to within 5 % only by completely combusting the sample and counting it as an infinitely thick layer of $BaCO_3$. In attempting to count thin layers of uncombusted compounds we have observed inaccuracies as high as 30 %.'

that if direct counting of organic samples is rejected as insufficiently accurate for a particular purpose then it is considerably easier to count carbon dioxide in a gas counter than to prepare barium carbonate samples for solid counting.

The high efficiency of gas counting shown in these results means that if metabolites are to be assayed by this method instead of by solid counting the dose of isotope used can be reduced by a factor of 10 or more. This represents a considerable saving of money as well as a tenfold reduction in health hazards and the precautions needed to combat them. This reduction in dose is also of considerable advantage in experiments where a physiologically excessive amount of labelled substrate would be required to produce metabolites sufficiently active for solid counting.

Flow counters: assay of tritium. Windowless flow-type counters have been described for the more efficient counting of solid samples. The sample is put inside the counter which is operated with a gas continually passing through it at atmospheric pressure. If helium containing a suitable quenching vapour is used the counter can be operated in the Geiger region (Kelsey, 1949; Damon, 1951). If

Table 3. *Comparison of ^{14}C solid counting at infinite thickness and gas counting of $^{14}CO_2$*

(For details of calculation see text.)

Substance	Activity of 20 mg. on 1 cm. disk (X) (counts/min.)	Vol. CO_2 from combustion of 20 mg. (V) (ml. at N.T.P.)	Specific activity of CO_2 (Y) (counts/min./ml.)	Total activity of V ml. (Z) (counts/min.)	Ratio Z/X = gain in efficiency	(Calc.) activity of C at infinite thickness ($=100A/BC$) (N) (counts/min.)	Ratio N/Y
Ca salts of mixed fatty acids containing ^{14}C	2439	24·6	4150	102 000	42	3620	0·87
[^{14}C]Glucose	120	15·0	450	6760	56	288	0·64
$Ba^{14}CO_3$	26	2·28*	320	730	28	329	1·03
$Ba^{14}CO_3$	2204	2·28*	29 300	66 700	30	29 000	0·99

* Liberated with acid.

The results in Table 3 are presented chiefly to demonstrate the much greater efficiency of gas counting compared with solid counting, and are not represented as the result of a rigorous comparative study of the two methods. It will, however, be noted that the ratios of the observed activity of the gas samples to the calculated activities of the carbon at infinite thickness are not the same for the few compounds listed. This suggests that, for these compounds in the particular equipment used, solid counting suffers from inaccuracies of the same order as those observed by Abrams & Clark (1951) and that a more detailed comparison of the two methods might be profitable. It is to be noted, moreover, methane is used the counter is operated in the proportional region (Eidinoff & Knoll, 1950; Sharpe & Taylor, 1951).

There is obviously a considerable gain in efficiency by this technique since window-absorption is eliminated and there is a more favourable geometry. It is particularly significant if solid tritiated compounds are to be counted since it is virtually impossible to make a window transparent to such low energy β-particles. Eidinoff & Knoll (1950) describe the counting of a tritiated steroid in their methane-flow type proportional counter. By spreading their sample in a thickness of 1 mg./sq.cm. over an area of 10 sq.cm. they were able to

count it with an efficiency of as much as 2·6 %. This is the infinite thickness value for tritium (0·8 mg./sq.cm.) and was obtained by evaporating a solution of the steroid. The authors observe that their technique will probably be applicable only to labelled substrates and that the metabolites obtained from biochemical systems will usually have such a low activity that they will have to be assayed by gas-counting methods. It is to be noted that if ^{14}C were present in the same material, solid counting would involve a rather complex procedure for the determination of both isotopes. On the other hand, by the combustion method described in this paper three isotopes can be determined with high efficiency and satisfactory accuracy as a routine procedure.

SUMMARY

1. A rapid method is described for the quantitative combustion of 10 mg. samples of biological materials.

2. Methods for the assay of tritium as tritiobutane and ^{14}C as $^{14}CO_2$ on this scale are briefly summarized, and methods of measuring and counting 10 μl.–10 ml. samples of radioactive gases are described.

3. Results are presented which demonstrate the quantitative combustion of some standard substances. The closely agreeing results of combusting and assaying in triplicate a sample containing the three isotopes are also presented.

4. The various methods of assaying the three isotopes are discussed with special reference to ^{14}C. The conclusions are drawn: (i) that gas counting is preferable for tritium especially at low activities and in the presence of ^{14}C; (ii) that the counting of solid samples containing ^{14}C is most reliable if first reduced to a single standard compound (usually $BaCO_3$); (iii) that gas counting is simpler than this and more efficient by at least a factor of 10, thus allowing a tenfold reduction in the dose of isotope used in biological experiments.

Thanks are due to Dr D. H. Tomlin, who carried out the ^{13}C assays in the Agricultural Research Council's mass spectrometer at Reading University, and to Mr B. W. E. Peaple for skilled technical assistance especially in the preparation and counting of tritiobutane.

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